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Original Article

Dual sgRNA-directed knockout *survivin* gene expression using CRISPR/Cas9 technology for editing *survivin* gene in triple-negative breast cancer

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (CRISPR/Cas9) offers a robust approach for genome manipulation, particularly in cancer therapy. Given its high expression in triple-negative breast cancer (TNBC), targeting *survivin* with CRISPR/Cas9 holds promise as a therapeutic strategy. The aim of this study was to design specific single guide ribonucleic acid (sgRNA) for CRISPR/Cas9 to permanently knock out the *survivin* gene, exploring its potential as a therapeutic approach in breast cancer while addressing potential off-target effects. *Survivin* gene knockout was conducted in the TNBC cell line BT549. Intron 1, exon 2, and intron 2 of the *survivin* gene were selected as sgRNA targets. These sgRNAs were designed in silico and then cloned into a CRISPR/Cas9 expression plasmid. The cleavage activity was assessed using an enhanced green fluorescent protein (EGFP) expression plasmid. The sgRNAs with higher cleavage activity were selected for the establishment of knockout cells. After transfecting the plasmid into the cells, the success of the *survivin* gene knockout was validated at the deoxyribonucleic acid (DNA) level using polymerase chain reaction (PCR) and sequencing analysis, and at the protein expression level using Western blotting. The study found that sgRNAs survin1A (targeting intron 1), survex2A (targeting intron 2), and survin2A (targeting intron 2) demonstrated higher cleavage activities compared to the other sgRNAs. However, using the single sgRNA, survex2A did not generate mutations in the *survivin* gene. At the protein level, survivin was still expressed, indicating that a single sgRNA was ineffective in knocking out the *survivin* gene. In contrast, the combination of sgRNA survin1A and sgRNA survin2A was more effective in generating mutations in the *survivin* gene, resulting in the deletion of the entire exon 2 and leading to a loss of survivin protein expression. In conclusion, our work provides specific sgRNAs and demonstrates the utilization of dual sgRNAs strategy in the CRISPR/Cas9 technology to knock out the *survivin* gene, showing potential in breast cancer therapy.

Keywords: CRISPR/Cas9, knockout, sgRNA, survivin, triple-negative breast cancer

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Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (CRISPR/Cas9) has emerged as a superior genome editing tool, outperforming zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) in terms of scalability, flexibility, and operability [1,2]. CRISPR/Cas9 technology comprises two main components: single guide ribonucleic acid (sgRNA) and Cas9 nuclease [2]. sgRNA, which includes mature CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA), directs the Cas9 protein to specific DNA targets, leading to double-strand breaks (DSBs) at these sites [3,4].

Protospacer-adjacent motif (PAM) is a short sequence in sgRNA used to identify target deoxyribonucleic acid (DNA) sites [5]. Cas9 cleavage induces DSBs approximately three base pairs upstream of the PAM site [6]. These breaks are typically repaired by non-homologous end joining (NHEJ), leading to indel mutations that can disrupt gene expression [7,8]. sgRNA can be engineered to adjust spacer length and sequence for increased specificity, directing Cas9 endonuclease to precise DNA locations and enhancing editing efficiency [9-12]. Potential off-target effects can undermine CRISPR/Cas9 efficiency, making meticulous sgRNA design essential during the initial stages of genome editing to ensure specificity and precision [13].

Survivin protein is highly expressed in various cancers, including breast cancer, and its elevated levels are associated with poor prognosis and resistance to chemotherapy and radiation [14,15]. Existing strategies to target *survivin* genes, such as the use of small molecules like sepantronium bromide (YM155) and small interfering RNA (siRNA), have demonstrated limitations in effectively addressing *survivin* gene overexpression [16,17]. Although both strategies have achieved transient downregulation of survivin, it requires repeated treatments to maintain suppression [18]. In contrast, gene editing of the baculoviral inhibitor of apoptosis proteins (IAP) repeat-containing 5 (*BIRC5*) gene, which encodes survivin, using CRISPR/Cas9 offers a promising and robust approach for genome manipulation, particularly in cancer therapy [19-21]. The aim of this study was to design specific sgRNA for CRISPR/Cas9 to permanently knockout the *survivin* gene, exploring its potential as a therapeutic approach in breast cancer while addressing potential off-target effects.

Methods

Study design and setting

This present study was an experimental in vitro study. *Survivin* gene knockout was conducted in the triple-negative breast cancer (TNBC) cell line BT549. Intron 1, exon 2, and intron 2 of the *survivin* gene were selected as sgRNA targets. The sgRNAs were designed in silico and then cloned into a CRISPR/Cas9 expression plasmid. The cleavage activity was assessed using an enhanced green fluorescent protein (EGFP) expression plasmid. After transfecting the plasmid into the cells, the success of the *survivin* knockout was validated using polymerase chain reaction (PCR) and sequencing analysis, and at the protein expression level using Western blotting. The schematic process of *survivin* knockout in the TNBC cell line using CRISPR/Cas9 technology is illustrated in **Figure 1**.

Cell culture

TNBC cell line BT549 was obtained from the American Type Culture Collection (ATCC) by the Laboratory of Experimental Pathology at the University of Tsukuba, Tsukuba, Japan. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 0.1 mg/mL streptomycin sulfate (Gibco Inc, New York, USA), and 10 µg/mL insulin (HiMedia Laboratories, Mumbai, India). Cultures were maintained at 37°C in a 5% CO_2 and 20% O_2 atmosphere.

Design of sgRNA and plasmid construction

Single guide RNAs (sgRNAs) targeting the *survivin* gene were designed in silico using the CRISPR Design Online Tool (https://crispr.dbcls.jp) [22]. Nucleotide sequences from intron 1 (5233–5484), exon 2 (5485–5594), and intron 2 (5595–7468) of the human *survivin* gene (NCBI

reference sequence NG_029069.1) were utilized for this design. The oligonucleotide sequences are detailed in **Table 1**. Survin1A and survin1B were sgRNAs targeting intron 1. Survin1A was located at nucleotide 5404–5426, while survin1B was located at nucleotide 5408–5429. Survex2A and survex2B were sgRNAs targeting exon 2. Survex2A was located at nucleotide 5511–5533. Meanwhile, survex2B was located at nucleotide 5513–5535. Survin2A and survin2B targeting intron 2, of which survin2A was located at nucleotide 6508–6530, meanwhile survin2B located in nucleotide 6719–6741.

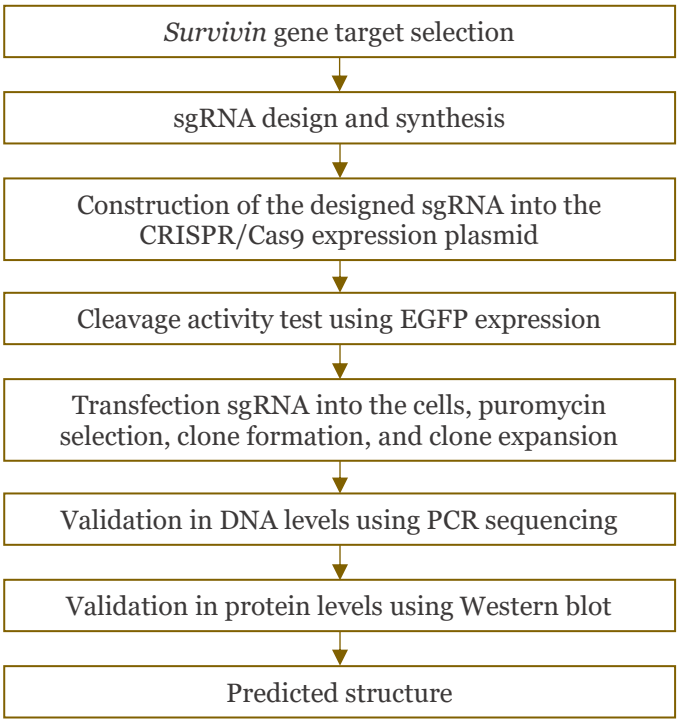

Figure 1. Schematic diagram of *survivin* gene knockout using clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (CRISPR/Cas9) in triple-negative breast cancer (TNBC) cell line BT549. EGFP: enhanced green fluorescent protein; PCR: polymerase chain reaction; sgRNA: single guide RNA

Forward and reverse oligonucleotides were phosphorylated and annealed (T4 polynucleotide kinase and T4 DNA ligase reaction buffer (New England Biolabs, Ipswich, Massachusetts, USA) and then inserted into pSpCas9(BB)-2A-Puro (PX459) V2.0 CRISPR/Cas9 cloning plasmid (Plasmid #62988, Addgene, Watertown, Massachusetts, USA) using *BbsI* restriction sites. This plasmid was transformed into *Escherichia coli* DH5α competent cells with 1 mg/mL ampicillin as the selectable marker [23]. Transformed cells were cultured on Luria-Bertani (LB) agar plates with 1 mg/mL ampicillin at 37°C. A single colony was used to inoculate LB medium with 1 mg/mL ampicillin, and the culture was incubated overnight at 37°C with shaking. Plasmid DNA was then purified using the Plasmid DNA Miniprep Purification Kit (Qiagen, Hilde, Germany).

Table 1. Oligonucleotide sequence of single guide RNA (sgRNA)

sgRNA	Sequence (5'→3')	
Survin1A	Forward	CACCGTGTCCCCATCGAGGCCTTTGTGG
	Reverse	AAACCCACAAAGGCCTCGATGGGGACAC
Survin1B	Forward	CACCGCCCATCGAGGCCTTTGTGGCTGG
	Reverse	AAACCCAGCCACAAAGGCCTCGATGGGC
Survex2A	Forward	CACCGCCCCACTGAGAACGAGCCAGACT
	Reverse	AAACAGTCTGGCTCGTTCTCAGTGGGGC
Survex2B	Forward	CACCGCCACTGAGAACGAGCCAGACTTG
	Reverse	AAACCAAGTCTGGCTCGTTCTCAGTGGC
Survin2A	Forward	CACCGTACTGCATCCCCGTAATCACTGG
	Reverse	AAACCCAGTGATTACGGGGATGCAGTAC
Survin2B	Forward	CACCGCCCTTGGGGAACCCGGGGCAATA
	Reverse	AAACTATTGCCCCGGGTTCCCCAAGGGC

Cleavage activity test

The cleavage activity test was conducted to evaluate the specificity of sgRNAs. The cleavage activity of each sgRNA was assessed using an enhanced green fluorescent protein (EGFP) system, as previously described [24]. sgRNA target sequences were amplified by PCR and inserted into the pCAG-EGxxFP EGFP expression plasmid (Plasmid #50716, Addgene, Watertown, Massachusetts, USA) [25], at the *BamHI* and *EcoRI* restriction sites. The plasmid was transformed into competent *E. coli* DH5α and purified using a Plasmid DNA Miniprep Kit (Qiagen, Hilden, Germany). Approximately 2 × 10^5 BT549 cells were seeded in a 6-well plate with 2 mL of medium. After overnight adaptation, 500 ng of pCAG-EGxxFP vectors containing the sgRNA target sequence and 500 ng of corresponding sgRNA expression vectors were transfected into the cells using polyethyleneimine (Sigma-Aldrich, Burlington, Massachusetts, USA). After 24 hours, EGFP fluorescence was visualized with a fluorescent microscope (Nikon, Tokyo, Japan), and fluorescence intensity was quantified using ImageJ version 1.53 (National Institutes of Health, Maryland, USA), with cells transfected with the control plasmid as a negative control. The fluorescence intensity was compared between survin1A and survin1B, survex2A and survex2B, as well as survin2A and survin2B.

Establishment of *survivin* gene knockout cells

BT549 cells were transfected with the most effective sgRNA expression vectors, selected based on cleavage efficiency, using polyethyleneimine (Sigma-Aldrich, Burlington, Massachusetts, USA). For single sgRNA experiments, 1 µg of sgRNA targeting exon 2 was used. In dual sgRNA experiments, 500 ng of each sgRNA targeting intron 1 and intron 2 were employed. Cells underwent puromycin selection (1 µg/mL) for three days, starting 24 hours post-transfection. After selection, cells were plated at 100 cells per 10 cm dish and cultured for two weeks to allow clone formation. Growing clones were then isolated for further expansion.

Screening for mutation by PCR and sequencing

To validate the success of genome editing at the DNA level, PCR and sequencing were conducted. DNA isolation was performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) following the manufacturer's instructions. PCR was carried out with MyTaq™ HS Red Mix (Bioline, London, UK) in a Veriti™ Thermal Cycler (Applied Biosystems, Life Technologies, Waltham, Massachusetts, USA), with an annealing temperature of 60°C. The PCR products were resolved on a 2% agarose gel, and bands of the expected sizes were excised and purified using a PCR Extraction and Purification Kit (Qiagen, Hilden, Germany). The purified products were sequenced using a DNA Sequencer 3130 (Applied Biosystems, Life Technologies, Waltham, Massachusetts, USA) with both forward and reverse primers. The primers used for PCR and sequencing are listed in **Table 2**.

Table 2. Primer sequence for PCR and sequencing

Primer	Sequence (5'→3')		Product size (bp)
Targeting sgRNA in exon 2	Forward (F1)	CCTGCTTTGTCCCCATCGAG	404
	Reverse (R1)	CCAAGGCATTTTGGTCAAATGAG	
Targeting sgRNA in intron 1 and intron 2	Forward (F2)	CAAGGACCACCGCATCTCTAC	1,469
	Reverse (R2)	AACATGGCCACACAAAGTCCA	

Western blotting

To validate the success of genome editing at the protein level, Western blot analysis was conducted. Wild-type and clone cell lysates were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (Abcam, Cambridge, UK) according to the manufacturer's instructions. Total protein concentration was assessed using Bradford assay (Bio-Rad, Hercules, California, USA) at 595 nm wavelength with a spectrophotometer. A total of 30 µg total protein was loaded onto a 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. Primary antibodies used included mouse anti-β-actin 1:4,000 dilution (Cell Signaling Technologies, Danvers, Massachusetts, USA) and rabbit anti-survivin 1:500 dilution (R&D Systems, Northeast Minneapolis, USA). Membranes were incubated with horseradish peroxide (HRP)-conjugated

secondary antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA) and protein bands were visualized using enhanced chemiluminescence (ECL) solution (Bio-Rad, Hercules, California, USA) with a chemiluminescence documentation system (UVITEC, Cambridge, UK). The intensity of each band was quantified using ImageJ version 1.53 (National Institute of Health, Maryland, USA). The band intensity from clone cells was compared to the band intensity from wild-type cells [21,24]; the intensity was presented in a unit of fluorescence (UF).

Protein prediction

Nucleotide sequences of *survivin* gene and amino acid sequences of survivin were retrieved from NCBI (NCBI Reference Sequence NG_029069.1 for nucleotide sequence and NP_001159.2 for amino acid sequence). The DNA-to-amino acid translation was performed using ExPASy online tools (https://www.expasy.org). Sequence alignment of nucleotides and amino acids was conducted with BioEdit software (North Carolina State University, North Carolina, USA). The predicted structure of knockout-survivin was modeled using Swiss-Model (https://swissmodel.expasy.org), and the 3D structure was visualized with PyMOL (Schrodinger Inc., New York, USA).

Study variables

In the cleavage activity assay, the dependent variable was the ratio of fluorescence intensity, while the independent variable was the sgRNA. Both variables consisted of numeric data. The cleavage activity was determined based on the comparison of mean values between survin1A and survin1B, survex2A and survex2B, and survin2A and survin2B. In the Western blot analysis, survivin protein expression was compared with wild-type cells for each.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) from three independent replicates per experiment. GraphPad Prism 9 was used for statistical analysis (GraphPad, Massachusetts, USA), with $p<0.05$ considered statistically significant. The difference between the wild-type and each clone (#1–#6) was determined using the independent Student t-test.

Results

Screening of sgRNA for *survivin* gene knockout

Six sgRNAs were designed using the *survivin* gene sequence spanning nucleotides 5233–7468 to induce mutations in exon 2 of the *survivin* genomic DNA. Two sgRNAs were selected from intron 1, exon 2, and intron 2 of the *survivin* gene sequences, as presented in **Figure 2**. The sequences of these sgRNAs, their positions in the *survivin* gene, and their on-target sites are detailed in **Table 3**. Cleavage activity tests were conducted to assess the efficiency of these sgRNAs in the TNBC cell line BT549.

Table 3. Sequences of single guide RNAs (sgRNAs)

sgRNA	Nucleotide position	Sequence (5'→3')	%GC of 20mer	Tm of 20mer (°C)	Number of target sites		
					20mer +PAM	12mer +PAM	8mer +PAM
Survin1A	(5404–5426) Intron 1	TGTCCCCATCGAG GCCTTTG**TGG**	60	77.59	1	1	6311
Survin1B	(5408–5429) Intron 1	**CCC**ATCGAGGCCT TTGTGGCTGG	60	78.31	1	1	435
Survex2A	(5511–5533) Exon 2	**CCC**CACTGAGAAC GAGCCAGACT	55	73.93	1	4	8182
Survex2B	(5513–5535) Exon 2	**CCA**CTGAGAACGA GCCAGACTTG	55	72.48	1	6	7294
Survin2A	(6508–6530) Intron 2	TACTGCATCCCCGT AATCAC**TGG**	50	72.86	1	2	2815
Survin2B	(6719–6741) Intron 2	**CCC**TTGGGGAACC CGGGGCAATA	60	80.60	1	2	7616

Bold nucleotides represent protospacer-adjacent motif (PAM) sequences; %GC: percentage of guanine and cytosine in the 20-nucleotide sgRNA sequence; Tm: melting temperature of the 20-nucleotide sgRNA sequence; 20mer+PAM, 12mer+PAM, and 8mer+PAM indicate exact matches of 20, 12, and 8 nucleotide sequences adjacent to the PAM site, respectively

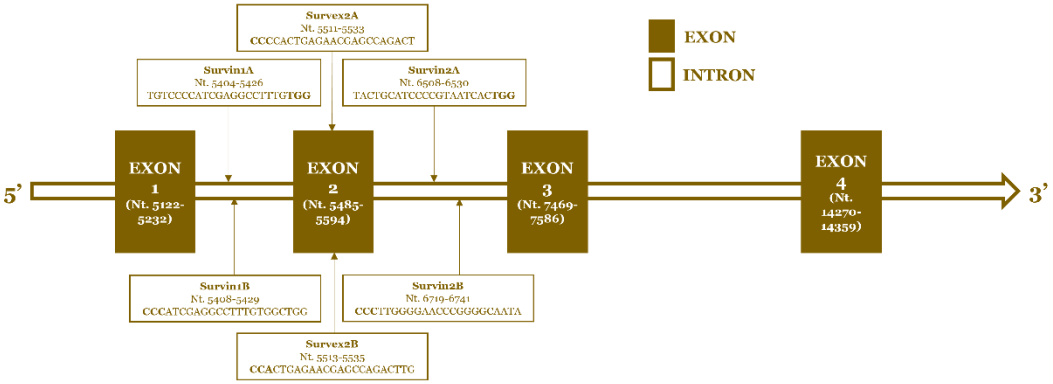

Figure 2. Schematic position of single guide RNAs (sgRNAs) targeted sequence of *survivin*. Two sgRNAs located in intron 1, exon 2, and intron 2 of the *survivin* sequence, respectively, were designed based on the on-target number.

All sgRNA-transfected cells displayed green fluorescence signals, confirming successful cleavage of the target site in the EGxxFP plasmid (**Figure 3**). Additionally, sgRNAs survin1A, survex2A, and survin2A exhibited stronger green fluorescence intensity compared to sgRNAs survin1B, survex2B, and survin2B, indicating higher cleavage activities. Consequently, sgRNAs survin1A (intron 1), survex2A (exon 2), and survin2A (intron 2) were selected for further applications.

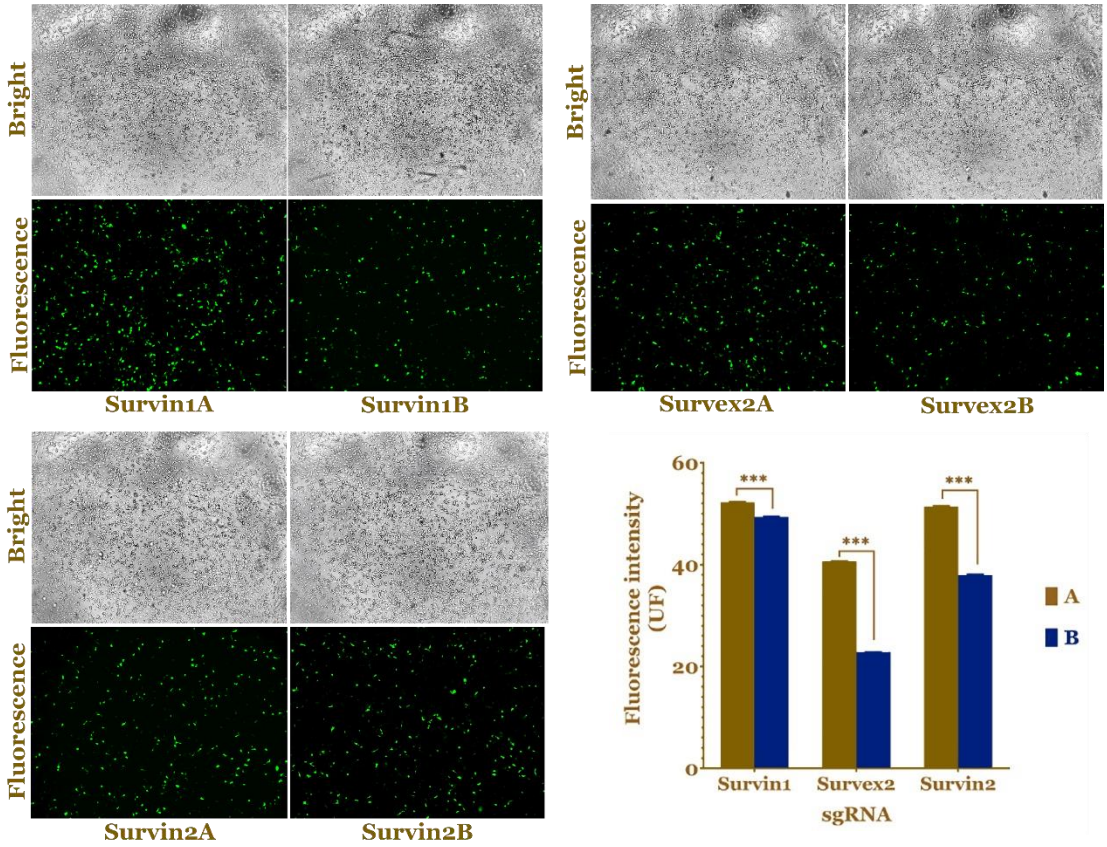

Figure 3. Cleavage activity assay of single guide RNAs (sgRNAs) in BT549 cell line. Fluorescence intensity was quantified by using ImageJ software. Data was presented as mean±SD of fluorescence intensity ratio to cells without sgRNA transfection (control). *** Significantly different at $p<0.001$. A in the figure refers to sgRNA survin1/2 or survex A, while B refers to sgRNA survin1/2 or survex B.

Single sgRNA for *survivin* gene knockout

Single sgRNA was employed to induce indel mutations in exon 2 of the *survivin* gene. sgRNA survex2A was transfected into BT549 cells, and following puromycin selection, six clones (#1–

#6) were expanded, then isolated and characterized by PCR using primers designed to amplify the sgRNA-targeted region. PCR products from the six clones were of similar size to those from wild-type cells, suggesting that the mutations might be small indels or nucleotide substitutions (**Figure 4A**). To verify the mutations, the PCR products were purified and sequenced using both forward (F1) and reverse (R1) primers. Alignment of the DNA sequences from all clones with those from wild-type cells revealed that sgRNA survex2A did not produce the expected mutations, as depicted in **Figure 4B**. Western blot analysis of all clones indicated that while survivin protein expression was reduced, it remained detectable, albeit at lower levels compared to wild-type cells (**Figure 4C**). These results suggest that a single sgRNA was ineffective in achieving a complete knockout of the *survivin* gene in the BT549 cell line.

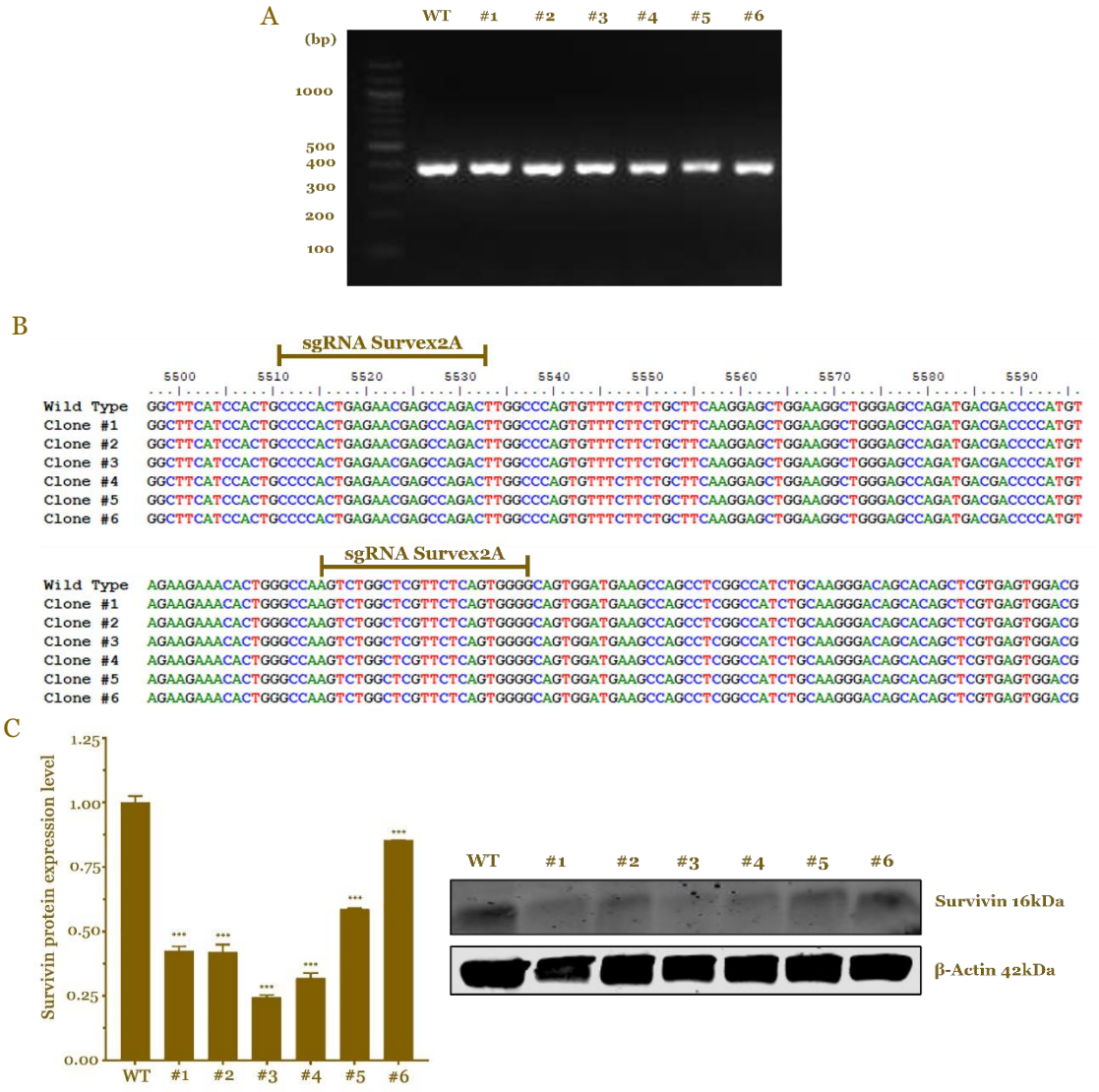

Figure 4. Establishment of *survivin* knockout in BT549 cells using single sgRNA. (A) PCR amplification of wild-type and 6 clones (#1–#6) in exon 2 of survivin gene targeted region. (B) Alignments of wild-type cells and all clones' sequences using forward primer (top) and reverse primer (bottom). (C) Survivin protein expression level in wild-type cells and all clones. Data was presented as mean±SD. *** Significantly different at $p<0.001$, where each clone was compared to wild-type cells as control.

Dual sgRNAs for *survivin* gene knockout

To achieve deletion of the entire exon 2 of the *survivin* gene, a combination of sgRNAs, survin1A (targeting intron 1) and survin2A (targeting intron 2), was utilized. Following puromycin selection, six clones were expanded, then isolated and characterized by PCR using primers flanking both sgRNA-targeted regions. PCR analysis, as presented in **Figure 5A,** revealed that

clones #1–#6 exhibited a lower band compared to the PCR products from wild-type cells (**Figure 5B**), indicating a deletion greater than 1,000 bp in the *survivin* gene. To determine the exact size of the deletion, the PCR products were purified and sequenced using forward primer (F2). Sequencing confirmed deletions of either 1,111 bp or 1,110 bp (**Figure 5C**). Protein expression analysis further confirmed the effectiveness of the dual sgRNA strategy, as all six clones showed a complete loss of survivin protein expression compared to wild-type cells (**Figure 5D**). These results demonstrate that the CRISPR/Cas9 genome editing technology successfully established a *survivin* gene knockout in BT549 cells by deleting the entire exon 2.

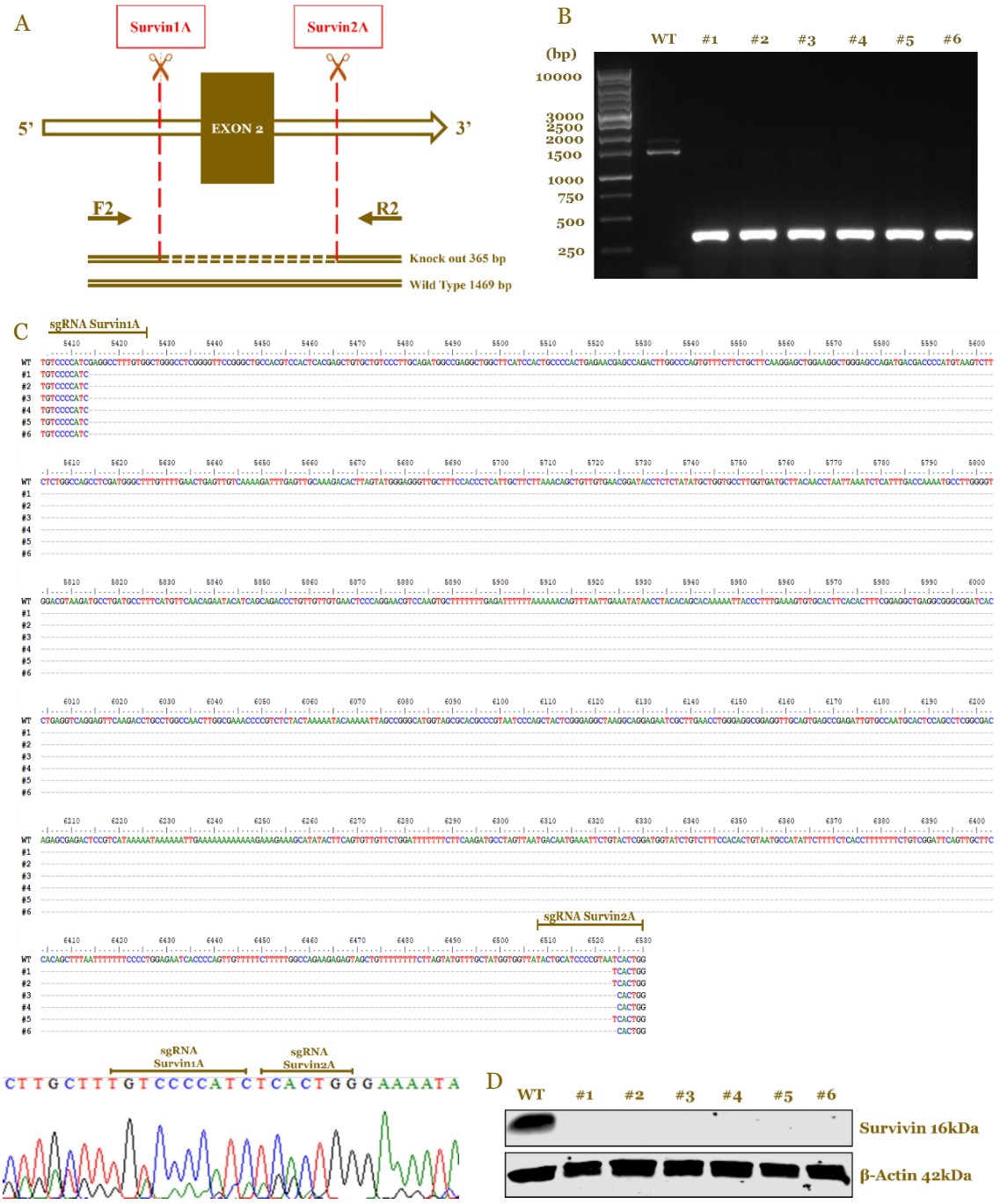

Figure 5. Establishment of *survivin* knockout in BT549 cells using dual single guide RNAs (sgRNAs). (A) Schematic of primer design to detect wild-type and knockout of *survivin* gene. (B) Polymerase chain reaction (PCR) amplification of wild-type cells and six clones (#1–#6) in exon 2 of *survivin* gene targeted region. (C) Alignments of wild-type cells and all clones' sequences show the success of *survivin* exon 2 deletion. A dash was used to symbolize the deletion of a single nucleotide. (D) Survivin protein expression level in wild-type cells and all clones. WT: wild type; KO: knockout; #1–#6: clone #1–#6.

Predicted structure of knockout survivin

Based on sequencing results, the impact of new nucleotide sequences on the three-dimensional protein structure and stability was assessed. According to the survivin amino acid sequence from NCBI Gene Bank (NP_001159.2), the wild-type sequence encodes 142 amino acids before reaching the stop codon UGA. In contrast, the successful survivin knockout DNA sequence translates to only 40 amino acids before encountering the stop codon UAA. **Figure 6A** illustrates that the alignment of amino acid sequences between wild-type and knockout shows only 26.76% similarity, with amino acids 1 through 37 being identical. The predicted three-dimensional structure (**Figure 6B**) reveals the loss of three helices and three β-sheets in the knockout variant.

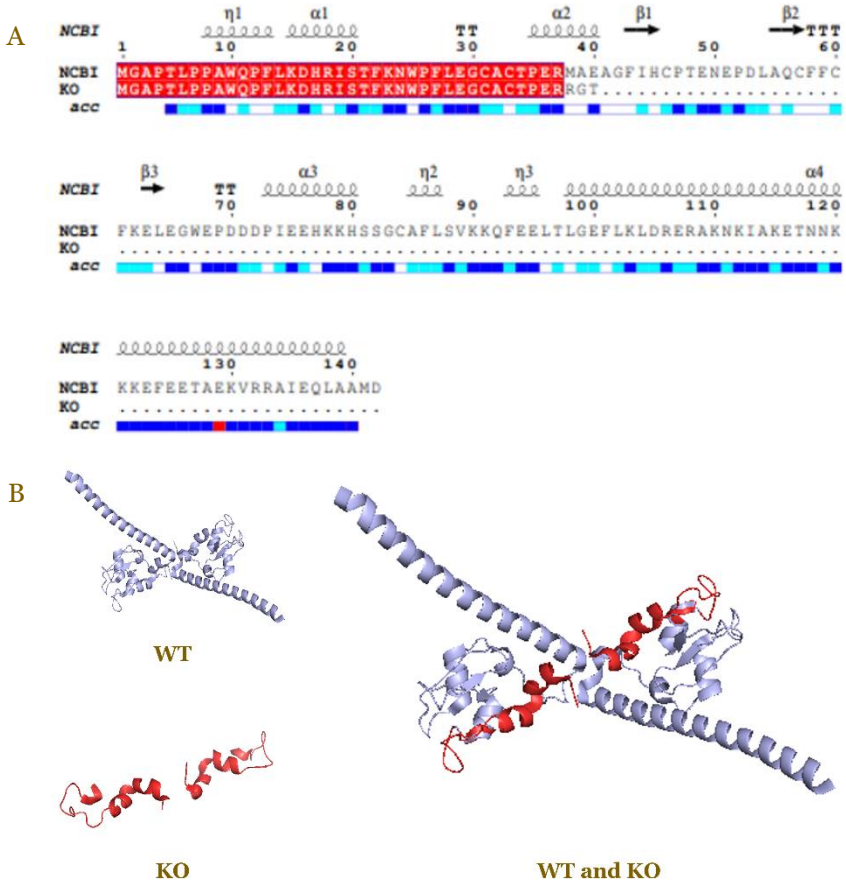

Figure 6. Predicted structure of wild-type and knockout survivin. (A) Alignment of knockout compared to wild-type amino acid sequence. (B) Predicted three-dimensional survivin protein structure of knockout compared to wild type. WT: wild type; KO: knockout.

Discussion

The feasibility of genome editing technology depends on the efficiency of regeneration following the editing process [26]. Optimizing techniques for regenerating and transforming specific varieties requires significant effort and resources [27]. Initial exploration of genome editing has led to various cloning strategies for diverse applications [28]. The creation of genome editing constructs relies on the potential of CRISPR/Cas9 plasmids, involving precise design and cloning methodologies [6]. Techniques to disrupt gene function can include single or multiple sgRNAs to induce base conversions, deletions, insertions, or combinations at targeted genomic locations [27]. The ability to edit genes offers a robust tool for precision and personalized therapy [4]. The present study highlights the design of sgRNAs for *survivin* genome editing, focusing on guanine and cytosine bases (GC) content, length, and the number of sgRNAs used. Dual-sgRNA targeting of *survivin* with CRISPR/Cas9 demonstrates potential as a novel alternative therapy for TNBC.

Elevated survivin expression can promote tumor progression through dysregulation of apoptosis, cell division, altered drug responsiveness, and enhanced cancer cell survival [14-17,29]. Thus, targeting survivin represents a promising therapeutic strategy for breast cancer, especially TNBC, which has limited treatment options [14,15]. The *survivin* gene produces several

splice variants, including survivin, survivin-2B, survivin-ΔEx-3, survivin-3B, and survivin-2-alpha, with exon 2 contributing to all isoforms [29]. Therefore, targeting exon 2 for knockout could effectively prevent the production of all survivin variants.

The present study employed two strategies: the first used a single sgRNA to induce mutations in exon 2, while the second utilized two sgRNAs to delete exon 2. The sgRNAs were selected based on a GC content of 50–60% and a maximum of one on-target value in the 20mer+PAM sequence. sgRNAs with 20 nucleotides and GC percentage within this range were chosen, as the present study suggested a correlation between GC content and cleavage efficiency. Previous study found efficient cleavage with GC percentages of 40–60% [30], while another study observed optimal cleavage with approximately 50% GC content [31]. Conversely, a previous study also reported decreased efficiency with GC content above 62.5% [32]. Moreover, the selection of sgRNA significantly impacts both the precision and effectiveness of CRISPR/Cas9 technology [33,34]. Another study demonstrated that sgRNAs with 17–20 nucleotides could achieve approximately 95% knockout efficiency in 293T cells [35].

Interestingly, 17-nucleotide sgRNAs may induce off-target effects, whereas 20-nucleotide sgRNAs do not, highlighting the importance of balancing on-target efficacy with off-target risks [35]. In the present study, on-target and off-target evaluations were based on the number of '20mer+PAM' and '12mer+PAM' matches. '20mer+PAM' and '12mer+PAM' refer to the exact matches of 20 and 12 nucleotides adjacent to the PAM site, respectively. A higher number indicates potential off-target sites, with a lower number preferable to minimize off-target editing. A score of '1' signifies a single exact match to the target site, while '0' indicates no matches in the genomic sequence [22].

CRISPR/Cas9 genome editing mechanism initiates by creating a DSB at the target site, which is then repaired via either homology-directed repair (HDR) or NHEJ [36]. HDR requires a homologous repair donor for precise modifications, while in the absence of such a donor, NHEJ repairs the DSB, leading to indel mutations [37]. Indel mutations in coding exons can introduce early stop codons or frame-shift mutations, resulting in protein activation [36,37].

Various strategies have been developed within the CRISPR/Cas9 system, including the use of one or multiple sgRNAs to induce deletions, insertions, base conversions, or other modifications at specific genomic locations [27]. One effective approach involves employing a pair of sgRNAs to generate two DSBs at a specific locus, facilitating the removal of the intervening DNA segment via NHEJ, which is advantageous for achieving biallelic modification [38]. This dual-sgRNA strategy also simplifies the visual identification of mutants through amplicon length analysis and has been shown to reduce off-target effects while improving editing efficiency, specificity, and accuracy [27,39,40]. Given that survivin is a small protein with limited sgRNA options for high on-target efficiency, the dual-sgRNA CRISPR/Cas9 strategy in the present study proved to be more effective for *survivin* knockout compared to single sgRNA approaches.

Genetic editing of target genes in breast cancer cells offers a powerful alternative to targeted therapy [4,6]. The present study proposes the use of dual-sgRNAs targeting *survivin* with CRISPR/Cas9 technology as a promising therapeutic approach for TNBC. However, this approach introduces complexities regarding sgRNA design challenge, recombination rate, and also potential off target effect. In this study, we have not yet explored off-target analysis in the region other than the *survivin* gene. Moreover, the effects of this knockout on cellular behavior, gene expression, and potential outcomes have not yet been explored. Therefore, further research is necessary to fully understand the impact of *survivin* knockout, particularly in the context of TNBC.

Conclusion

Genome editing can be implemented as an alternative for targeted therapy in breast cancer treatment. Our results provide specific sgRNAs for *survivin* gene editing. Moreover, we have successfully proved that dual-sgRNAs are a better choice than single-sgRNA for *survivin* gene editing. To the best of our knowledge, this present study is the first to apply CRISPR/Cas9 technology with a dual-sgRNAs strategy for knocking out the *survivin* gene in TNBC, demonstrating significant potential for TNBC treatment.

Ethics approval

The protocol of the present study was reviewed and approved by the Ethical Committee of Health Research, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia (Approval number: 801/UN2.F1/ETIK/PPM.00.02/2022).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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