International Journal of Technology 15(2) 353-363 (2024)
Received July 2023 / Revised September 2023 / Accepted October 2023

International Journal of Technology

# Design of Specific and Efficient sgRNA for CRISPR/Cas9 System to Knockout Superoxide Dismutase 2 in Breast Cancer Stem Cells

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**Abstract.** This study aimed to design specific and efficient single guide RNA (sgRNA) for CRISPR/Cas9 system to knockout human superoxide dismutase 2 (SOD2) in human breast cancer stem cells (BCSCs). To achieve this, two sgRNA targets were selected, located within the Ala16Val polymorphism and the conserved region of human SOD2 variants. Design process was carried out using CRISPRdirect tool, considering the on/off target efficiency score. Subsequently, these sgRNAs were cloned into CRISPR/Cas9 expression plasmid and transfected into both the CD24-/CD44+ and ALDH1+ human BCSCs. To determine the most efficient sgRNA, a cleavage activity assay was conducted. The effectiveness of CRISPR/Cas9 system in knocking out the mRNA and protein expression of SOD2 in BCSCs was determined using quantitative reverse transcriptase polymerase chain reaction and western blot assays, respectively. The results showed that the sodex2.1 sgRNA targeting the Ala16Val region within exon 2 was ineffective in knocking out the SOD2 protein expression. However, among the four sgRNAs targeting the conserved region of nine SOD2 variants, the sodex2.4 sgRNA spanning from nucleotide 532-554 showed the highest efficiency based on cleavage activity assays. The sodex2.4 sgRNA significantly decreased both mRNA and protein expressions of SOD2 in human BCSCs. In conclusion, this study successfully designed specific and efficient sgRNA to knockout SOD2 expression in human BCSCs using CRISPR/Cas9 system. Moreover, further investigations are recommended to understand the impact of SOD2 knockout on the aggressiveness of breast cancer, particularly in BCSCs.

*Keywords:* Breast cancer; CRISPR/Cas9; sgRNA; SOD2; Stem cells

## 1. Introduction

Tissue engineering is currently playing a significant role in the rapid progression of various technologies in medical applications (Irsyad *et al.,* 2022). The integration of gene editing strategies into tissue engineering, necessitating nuclease modifications is essential

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doi: 10.14716/ijtech.v15i2.6680

in the treatment of numerous genetic diseases. Among the available genome editing system, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) associated nuclease 9 (Cas9) is the most potent option, characterized by low off-target effects (Atkins *et al.,* 2021; Aryal, Wasylishen, and Lozano. 2018). CRISPR/Cas9 system depend on a single guide RNA (sgRNA), consisting of CRISPR RNA (crRNA) and trans-activating (tracrRNA), to cleavage specific gene sites. The significance of sgRNA sequences is closely associated with target specificity and the cleavage efficiency of the Cas9 enzyme at the target site (Malik *et al.,* 2021). However, off-target effects can occur when sequences similar to sgRNA are present in other parts of the genome, leading to unintended gene knockout (Modrzejewski *et al.,* 2020). In the process of designing specific and efficient sgRNAs, there is a possibility of predicting their efficiency by considering various factors such as GC content, off-target score, and sequence motifs, correlated with gene target features (Konstantakos *et al.,* 2022).

Cancer is a multifaceted disease characterized by the unregulated growth of abnormal cells, originating from cancer stem cells (CSCs). These CSCs form a side population with stemness properties similar to normal stem cells, possessing high tumorigenicity which contributes to the progression of cancer (Ahmad, Zain, and Aziz, 2018; Ayob and Ramasamy, 2018). Among all cases, breast cancer is the most frequently diagnosed type, ranking as the first leading cause of cancer-related death in women globally (Nusantara *et al.,* 2016). Previous studies have identified several CSC populations in human breast cancer, including CD24-/CD44+ and aldehyde dehydrogenase-1 (ALDH1+) cells (Shiraishi *et al.,* 2017; Wanandi *et al.,* 2017). The presence of breast cancer stem cells (BCSCs) strongly correlates with biological aggressiveness, resulting in a poorer prognosis (Sarkar *et al.,* 2018).

Several studies have reported that the aggressiveness of BCSCs is closely correlated with the overexpression of superoxide dismutase 2 (SOD2), a mitochondrial antioxidant enzyme. SOD2 plays a critical role in safeguarding cells from excessive superoxide, the primary reactive oxygen species (ROS) produced within the mitochondria of cells (Azadmanesh, Trickel, and Borgstahl, 2017). Initially regarded as a tumor suppressor, decreased SOD2 expression was associated with cellular transformation and tumorigenesis due to high ROS-mediated DNA damage, resulting from the accumulation of superoxide and other oxidants. However, recent research has shown that the overexpression of SOD2 in cancer cells induces a moderate increase in sub-lethal $H_2O_2$, triggering oxidation and enhancing redox signalling. This phenomenon promotes cells proliferation and increases cancer aggressiveness, showing the dual role of SOD2 in tumorigenesis (Kim *et al.,* 2017).

The SOD2 gene is located on chromosome 6q35, comprising 5 exons and 4 introns. In this gene, a well-studied single nucleotide polymorphism (SNP) known as Ala16Val in exon 2 causes changes in the 16th amino acid from valine to alanine. This SNP has been correlated with the changes in SOD2 conformation, activity in mitochondria, and risk of various diseases, including breast cancer (Sari *et al.,* 2019; Wang *et al.,* 2018; Abdelrauf, et al., 2017).

CRISPR/Cas9 gene editing technology has been applied to knockout SOD2 expression in HEK293T using sgRNA targeting exon 3 of the SOD2 gene (Cramer-Morales *et al.,* 2015). The impact of this technology is limited to specific SOD2 variants that possess an intact exon 3. However, there is a lack of data regarding specific SOD2 variants within human BCSCs contributing to their aggressiveness. Consequently, this study aimed to design specific and efficient sgRNA for CRISPR/Cas9 system, targeting the Ala16Val SNP and the conserved region common to all SOD2 variants to knockout SOD2 expression in breast cancer cells, particularly the most aggressive BCSCs.

## 2. Methods

### *2.1. Cells Culture*

ALDH1+ and CD24-/CD44+ BCSCs were obtained from Cells Culture Laboratory for Cancer Stem Cells, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. These cells were cultured in a serum-free DMEM/F12 medium (Gibco, Thermo Fisher Scientific Inc., Massachusetts, USA). Furthermore, the HEK293T cells line, derived from a human embryonic kidney cells line, was sourced from ATCC or Riken Bioresource Research Center (Wardhani *et al.,* 2020; Amalia *et al.,* 2019) and maintained in DMEM (Gibco™, Thermo Fisher Scientific Inc., Massachusetts, USA) supplemented with 10% Fetal Bovine Serum (Gibco™, Thermo Fisher Scientific Inc., Massachusetts, USA), penicillin G (Gibco™, Thermo Fisher Scientific Inc., Massachusetts, USA), streptomycin sulfate (Gibco™, Thermo Fisher Scientific Inc., Massachusetts, USA), and 10 µg/mL of insulin. All cells line were incubated at $37^0$C with 5% $CO_2$ and 20% $O_2$.

### *2.2. CRISPR/Cas9 Genome Editing*

This study has been granted ethical approval by the Ethics Committee of the Faculty of Medicine, University of Indonesia - Cipto Mangunkusumo Hospital, with number KET-B64/UN.2.F1/ETIK/PPM.00.02/2022. The schematic process of CRISPR/Cas9 genome editing of the SOD2 gene is illustrated in Figure 1.

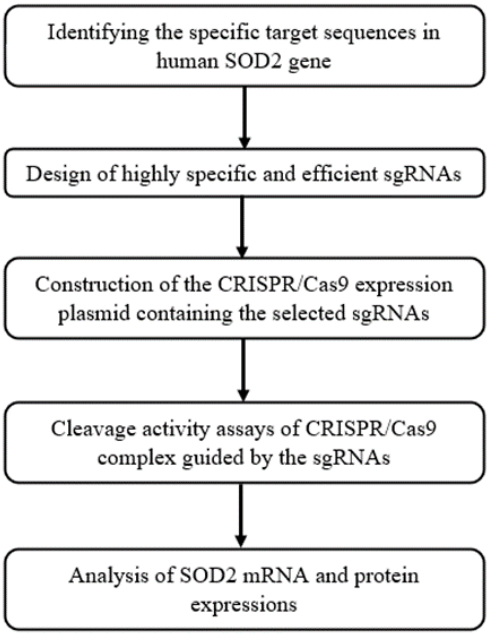


**Figure 1** Flowchart of CRISPR/Cas9 genome editing of the SOD2

#### 2.2.1. sgRNA Design

The initial design of sgRNA targeting SOD2 in exon 2 was carried out using CRISPRdirect website computational tool (https://CRISPR.dbcls.jp/)(Naito *et al.,* 2014) based on the NCBI database (NG_007829.4) (NCBI, 2023).

#### 2.2.2. Construction of CRISPR/Cas9 Plasmid

Designed sgRNA sequences were inserted into CRISPR/Cas9 cloning plasmid pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene, plasmid #62988, Watertown, USA) using BbsI restriction sites. These sgRNA sequences were introduced into competent *E. coli* DH5α cells through 1 mg/mL ampicillin selection. Bacterial cultures were grown on LB agar plates at 37°C, followed by growth in LB broth within a shaking incubator at 37°C. Subsequently,

plasmids were extracted using a High-speed Plasmid Mini Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan).

2.2.3. Cleavage Efficiency Assay

Cleavage efficiency of each sgRNAs was assessed using the enhanced green fluorescent protein (EGFP) expression plasmid (pCAG-EGxxFP) (Addgene, plasmid #50716, Watertown, USA) in HEK293Tcells, as previously described (Wardhani *et al.,* 2020). The SOD2 target sequences were ligated between EGFP fragments of pCAG-EGxxFP plasmid and transformed into E. coli DH5a competent following the protocol of the manufacturer. The resulting target plasmid was purified using a Miniprep purification kit (Qiagen Ltd., Manchester, UK). Subsequently, the purified plasmid was transfected with CRISPR/Cas9 plasmid expressing sgRNA SOD2 gene and hCas9 into human cells using polyethylenimine (Sigma-Aldrich Chemie GmbH, Taufkirkchen, Germany). The EGFP in transfected cells was observed with a fluorescence microscope (Eclipse Ni-U with Intenslight, Nikon Instruments Inc., New York, USA). Quantification of fluorescence intensity was measured by ImageJ version 1.53, compared to negative plasmid control without sgRNA expression vector.

2.2.4. Preparation of Total RNA and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were extracted using Tripure Isolation Reagent (Roche Applied Science, Basel, Switzerland) based on the guidelines of the manufacturer, while concentrations were quantified with a spectrophotometer. For qRT-PCR, SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline, Meridian Bioscience, London, UK) was conducted in a Real-Time PCR System 7500 Fast Thermal Cycler (Applied Biosystems, Life Technologies, California, USA), according to the protocol of the manufacturer. Primer sequences for 18S rRNA were 5'-AAACGGCTACCACATCCAAG-3' (forward) and 5'-CCTCCAATGGATCCTCGTTA-3' (reverse), while SOD2 consisted of 5'-GCACTAGCAGCATGTTGAGC-3' (forward) and 5'-ACTTCTCCTCGGTGACGTTC-3' (reverse). Each reaction was performed in triplicate and the relative mRNA expression levels were determined using the Livak formula (Livak and Schmittgen, 2001).

2.2.5. Western Blot Analysis of SOD2

Total protein was extracted using RIPA Lysis Buffer (Abcam, Cambridge, UK) and protein concentration was measured using Bradford Assay (Bio-Rad Laboratories Inc., California, USA). Equal amounts of protein (20 µg/lane) were loaded onto 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Primary antibodies used included rabbit anti-SOD2 1:1000 (Cells Signalling Technologies, Massachusetts, USA) and mouse anti-β-actin 1:2000 (Cells Signalling Technologies, Massachusetts, USA). To enhance visualization, chemiluminescence reagent (Abcam, Cambridge, UK) was used, while the blots were captured using Chemiluminescence Documentation System (UVITEC Ltd., Cambridge, UK).

*2.3. Statistical analysis*

All data presented were expressed as means ± standard deviation from three independent experiments. Knockout data were compared to WT (wildtype) cells without genome editing, and statistical analysis was performed using an independent t-test.

## 3. Results and Discussion

SOD2 has been reported to play dual and contradictory roles in tumorigenesis, acting as a tumor suppressor by mitigating oxidative stress in the early stage of carcinogenesis. Furthermore, it serves as a tumor promotor that stimulates cells proliferation and metastasis by suppressing ROS-induced cytotoxicity in malignant tumors (Wanandi *et al.,*

2017). In a previous study, Morales et al. used CRISPR/Cas9 genome editing to target SOD2 in HEK293T cells with sgRNA located in exon 3. Consequently, cells lacking SOD2 showed reduced clonogenic potential due to impaired mitochondrial function resulting from increased oxidative stress (Cramer-Morales *et al.,* 2015). Although SOD2 knockout studies have been carried out in human cells, there is no report on cancer cells, particularly in BCSCs.

Several studies have showed that BCSCs show higher levels of SOD2 compared to the non-BCSC population. This high expression strongly correlates with their aggressiveness, including stemness and metastasis (Srivastava *et al.,* 2023; Wanandi *et al.,* 2019). However, specific sequence variation in human SOD2 contributing to the aggressive properties remains unknown. Previous investigations have shown that the Val/Val genotype shows low SOD2 activity. Meanwhile, the Ala/Ala genotype, which expresses a high SOD2 level, is significantly associated with increased cancer risk (Da-Cruz-Jung *et al.,* 2020; Wang *et al.,* 2018). This SNP is not present in any of the nine SOD2 variants published in the NCBI database (NCBI, 2023). Consequently, to knock out the SOD2 gene in human BCSCs using CRISPR/Cas9 system, two sgRNA targets were used in this study. These included one sgRNA target located within the Ala16Val polymorphism region within exon 2 and the other in the conserved region common to all SOD2 variants.

In this study, sgRNAs were designed using CRISPRdirect that can precisely identify on-target locations, while minimizing the possibility of off-target effects (Karlapudi *et al.,* 2018; Naito *et al.,* 2014). The selected sgRNA has a single exact match with the target sequence, specifically designed for editing SOD2 in BCSCs. To target the Ala16Val site at nucleotide (nt) 401-403, sgRNA was designed spanning from nt 385-407 within exon 2 of the SOD2 gene, as presented in Figure 2A. Before transfecting CRISPR/Cas9 plasmid containing the sodex2.1 sgRNA into BCSCs, its efficiency was assessed in a cleavage activity assay using HEK293T cells transfected with a constructed pCAG-EGxxFP expression plasmid. The result showed a moderate green fluorescence intensity produced from EGFP expression, as presented in Figure 2B. This suggested moderate efficiency of sgRNA in guiding Cas9 to cleave the DNA target. Subsequently, the effectiveness of the sodex2.1 sgRNA for CRISPR/Cas9 system was evaluated by analyzing SOD2 expression in HEK293T cells, as depicted in Figure 2C. Although the relative mRNA expression decreased to 0.6-fold, Cas9 cleavage had no significant impact. This suggested that sgRNA targeting the Ala16Val SNP region was ineffective in knocking out the SOD2 expression in HEK293T cells due to the absence of the Ala16Val site in these cells.

Despite the careful selection of sgRNA based on its high specificity, the results showed low efficacy. Based on previous study, Cas9-mediated cleavage can be nullified by single mismatches occurring at the interface between sgRNA and the target site. This effect is predominant within the final 10-12 nucleotides situated at the 3' end of the 20-nucleotide sgRNA targeting region (Zhang *et al.,* 2017). The decrease in mRNA expression following CRISPR/Cas9 gene editing is attributed to various factors, including alterations in transcriptional regulation, mRNA stability, or degradation sites (Bishop and Hawley, 2022; Javaid and Choi, 2021). The altered codons might not have been translated into different amino acids or influenced the SOD2 conformation, causing challenges in its detection in a western blot assay.

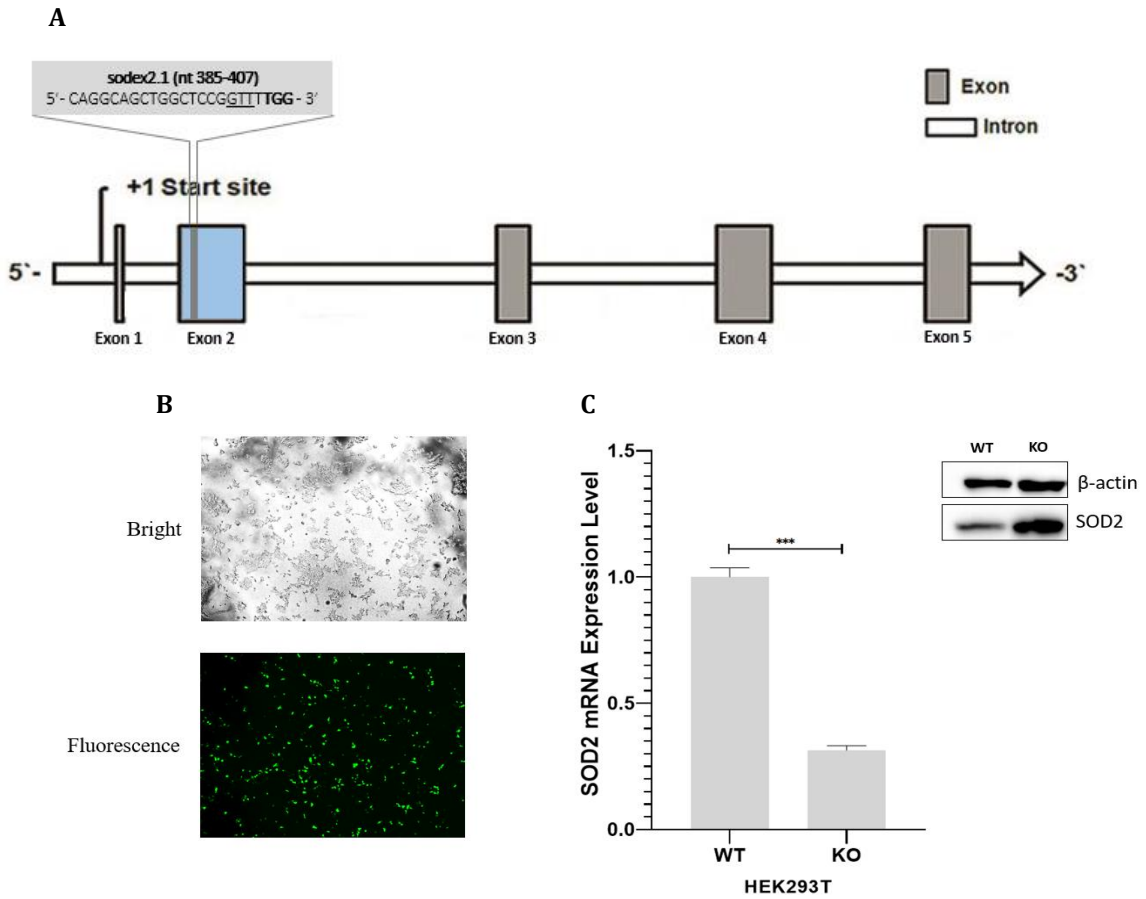


**Figure 2** Design of sgRNA targeting the Ala16Val SNP region within exon 2 of the SOD2 gene. (A) Selected sodex2.1 sgRNA sequence from exon 2 (blue box), which contains Ala16Val site in nt 401-403 (underlined codon); PAM of the target, bold fonts. (B) Cleavage activity assay in HEK293T cells showed moderate green fluorescence intensity. (C) Reduction in relative mRNA expression, while the protein expression level remained unaffected, showing no significant impact, ***p<0.001.

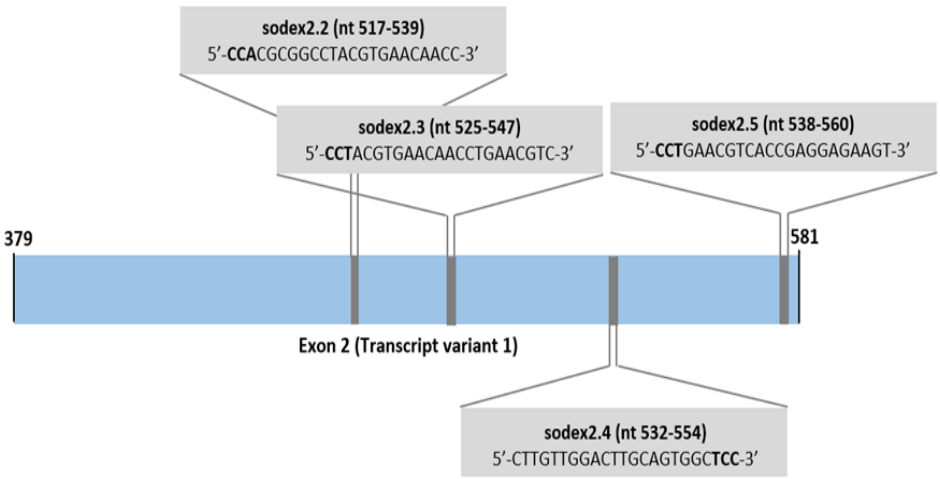


**Figure 3** Position of sgRNAs targeting the conserved region of SOD2 variants within exon 2. Four sgRNAs were selected based on the efficiency score (max. efficiency = 1). sgRNAs sequences, grey boxes; exons, blue box; PAM of the target, bold fonts

To ensure the effectiveness of CRISPR/Cas9 system across all SOD2 variants, sgRNAs targeting the conserved region among nine SOD2 variants as identified in the NCBI database, were designed as presented in Figure Supplementary. A total of four sgRNA

candidates were obtained using CRISPRdirect online tools, described in Figure 3, providing information about their respective on-target sites, as shown in Table 1. According to a previous study, a greater number of on-target sites showed a higher potential for off-target effects (Naito *et al.,* 2014). The characteristics of sgRNAs, including %GC content, sgRNA length, and melting temperature also play crucial roles in determining on-target efficacy. Previous studies have shown that sgRNAs with a GC content ranging from 40 to 60% are effective in enhancing gene editing efficiency using CRISPR/Cas9 system. Specifically, sgRNAs with approximately 50% GC content are recommended due to their efficiency in CRISPR gene editing (Mohammadhassan *et al.,* 2022). Lv *et al.* (2019) also showed that a 20-nucleotide segment of sgRNA can be highly effective in reducing off-target effects. However, when sgRNA is shorter than 15 nucleotides, the Cas endonuclease may not show any activity for knocking out the target gene (Lv *et al.,* 2019). In this study, all sgRNAs were approximately 20 nucleotides in length, consisting of only one target site within the 20mer+PAM, which showed a high level of specificity.

**Table 1** Characteristics of four sgRNA candidates

| sgRNA name | Position | | %GC | Tm (°C) | Number of target sites | | |
|---|---|---|---|---|---|---|---|
| | start | end | | | 20mer+PAM | 12mer+PAM | 8mer+PAM |
| sodex2.2 | 517 | 539 | 65 | 78.95 | 1 | 1 | 1285 |
| sodex2.3 | 525 | 547 | 50 | 70.89 | 1 | 3 | 789 |
| sodex2.4 | 532 | 554 | 55 | 72.29 | 1 | 2 | 429 |
| sodex2.5 | 538 | 560 | 55 | 73.54 | 1 | 2 | 734 |

Computational tools offer valuable insights into sgRNA specificity. However, verifying their specificity and efficiency through cleavage activity assay is essential. Among the four sgRNAs tested, sodex2.4 showed the highest cleavage activity on the pEGxxFP expression plasmid. This is evident by the strongest green fluorescence intensity of EGFP presented in Figures 4A and 4B. This observation corresponds with the assessment conducted using CRISPRdirect tool, which identified sodex2.4 as the optimal sgRNA based on the number of on-target sites and GC content. Consequently, the effectiveness of sodex2.4 sgRNA in suppressing SOD2 expression in HEK293T cells was validated. The results showed a complete disruption of SOD2 mRNA expression to 0.3-fold and approximately complete loss of its protein expression. This showed knockout effect of SOD2 expression following CRISPR/Cas9 genome editing, illustrated in Figures 4C and 4D.

Further assessment was conducted to evaluate the optimal potential of sodex2.4 sgRNA to knockout SOD2 expression in CRISPR/Cas9 genome editing experiments conducted on human BCSCs, specifically ALDH1+ and CD24-/CD44+ cells. The results showed a significant reduction in SOD2 mRNA expression in CD24-/CD44+ (C#1) and ALDH1+ (A#1 and A#2) clones reaching levels of 0.3, 0.25, and 0.05, respectively, compared to wild-type cells, as presented in Figure 5A. These results were corroborated by protein expression analysis through Western Blot assays shown in Figure 5B. As anticipated, all KO-SOD2 clones in CD24-/CD44+ and ALDH1+ cells showed values ranging from very low to negligible expression of SOD2 protein. This showed the successful knockout of SOD2 expression at both mRNA and protein levels in human BCSCs.

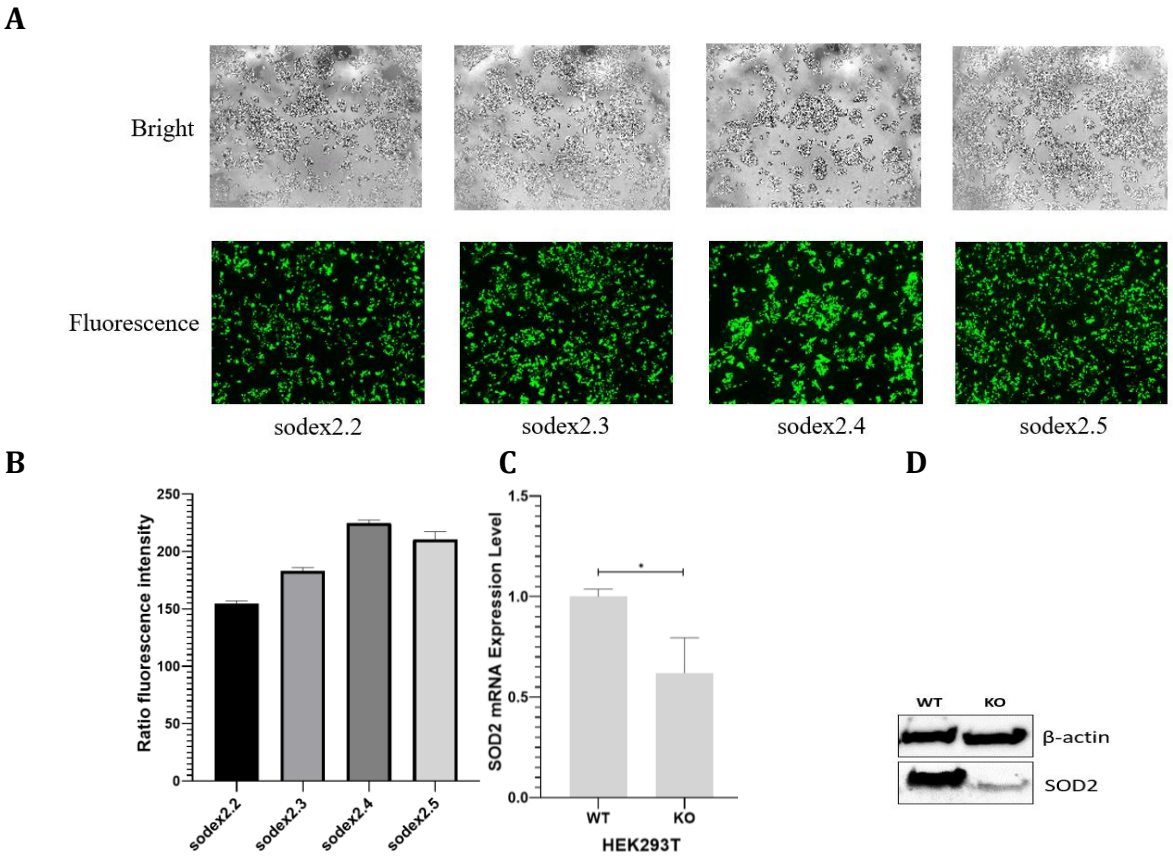


**Figure 4** The efficiency of sgRNAs targeting the conserved region of all SOD2 variants for CRISPR/Cas9 system in HEK293T cells. (A-B) Cleavage activity assay. (A) Fluorescent microscope images were contrasted with cells under a bright field. (B) Fluorescence intensity ratio of HEK293T cells edited with sodex2.2, sodex2.3, sodex2.4, and sodex2.5 CRISPR/Cas9 system to wild-type HEK293T cells. Fluorescence intensity was determined using ImageJ software and data obtained were represented as mean ± SD. (C) SOD2 mRNA relative expression levels in HEK293T cells edited with sodex2.4 CRISPR/Cas9 system normalized to wild-type (WT) HEK293T. *p<0.01. (D) SOD2 protein expression in HEK293T cells edited with sodex2.4 CRISPR/Cas9 system compared to WT HEK293T and β-actin.

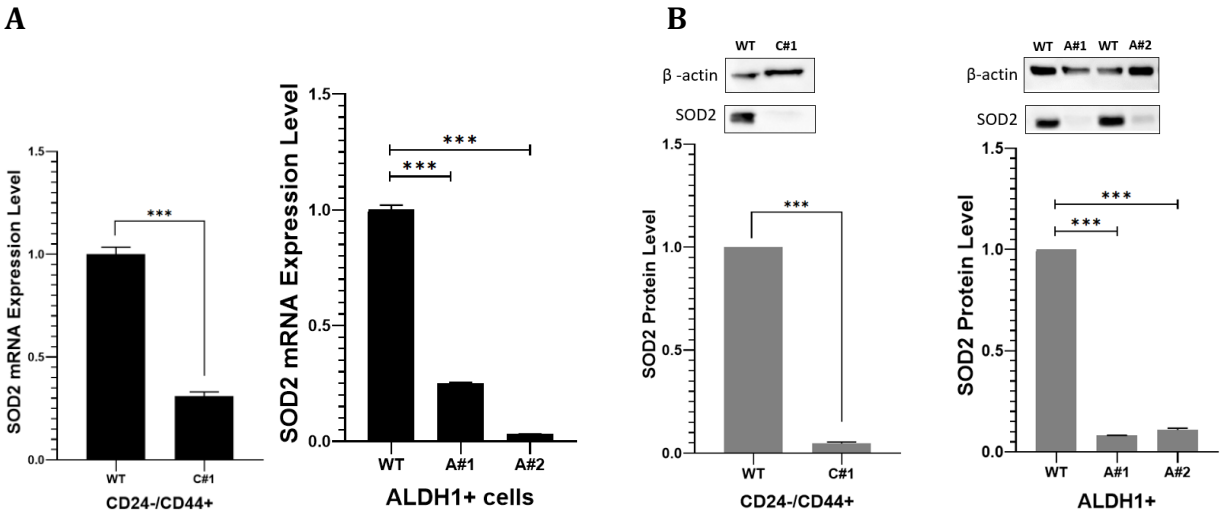


**Figure 5** Effect of CRISPR/Cas9 genome editing of SOD2 in human CD24-/CD44+ and ALDH1+ BCSCs. (A) mRNA expression levels of SOD2 in BCSCs were analyzed using qRT-PCR. (B) Protein expression of SOD2 in BCSCs analyzed using Western Blot assay. WT

stands for wild-type, C#1, A#1, and A#2 were SOD2 knockout (SOD2-KO) clones in the respective BCSCS. β-actin was used as a housekeeping protein. All data of SOD2-KO were compared to the wild-type cells, without genome editing, ***p<0.001.

## 4. Conclusions

CRISPR/Cas9 system is widely acclaimed in the field of genetic engineering for its precision, effectiveness, and affordability in genome editing. However, design of specific and efficient sgRNA remains a challenging endeavor, requiring careful consideration of gene variations. In conclusion, we successfully designed four sgRNAs to knockout the SOD2 gene expression in BCSCs using CRISPR/Cas9 technology. Among these four sgRNAs, we chose sgRNA sodex2.4, which has the highest efficiency of cleavage activity, to verify the knockout effect at the mRNA and protein levels. These findings highlight that precise sgRNA design is important to specifically target the CRISPR/Cas9 genome editing. Further studies are needed to elaborate the impact of KO SOD2 on the aggressiveness of BCSCs.

## Acknowledgments

This study received support from the PUTI Q2 Batch 2 Research Grant (Number: KB-1252/UN2.RST/HKP.05.00/2022) provided by the Directorate for Research and Community Engagement at Universitas Indonesia.

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