FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 60, No. 1, 2022
pp. 13–23

ORIGINAL PAPER

VIA MEDICA

Plasmid-based CRISPR-Cas9 system efficacy for introducing targeted mutations in CD81 gene of MDA-MB-231 cell line

Kasra Arbabi Zaboli[1*], Hossein Rahimi[1*], Jose Thekkiniath[2], Amir Hossein Taromchi[1], Saeed Kaboli[1, 3]

[1]Department of Medical Biotechnology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran
[2]Fuller Laboratories, Fullerton, CA, USA
[3]Cancer Gene Therapy Research Center, Zanjan University of Medical Sciences, Zanjan 4513956111, Iran
*Kasra Arbabi Zaboli and Hossein Rahimi equally contributed to this work

Abstract

Introduction. Breast cancer has been represented a challenging issue worldwide as it is one of the major leading causes of death among women. CD81 gene, a member of the tetraspanin protein family, has been associated with the development of human cancers. Genome editing technologies, particularly the CRISPR-Cas9 system, have shown rapid progress in gene function studies. In this study, we aimed to evaluate the ability of the CRISPR-Cas9 plasmid-based system to modify specific regions of the CD81 gene in the MDA-MB-231 breast cancer cell line.

Materials and methods. Using bioinformatics database search, four different single guide RNAs (sgRNAs) to target exon 3 and exon 5 of the CD81 gene were designed. The intended sgRNAs sequences were cloned into the expression plasmid pSpCas9(BB)-2A-GFP (PX458) bearing sgRNA scaffold backbone, Cas9, and EGFP coding sequences, which was confirmed by colony PCR and sequencing. Transfection efficiency was determined by fluorescence microscopy and flow cytometry analysis. Gene editing efficiency was measured qualitatively and quantitatively using the T7E1 and TIDE software, respectively.

Results. Our data show that expression constructs were successfully introduced into MDA-MB-231 cells with an acceptable transfection efficiency. Two sgRNAs that were afforded to introduce significant mutations in their target regions were detected by TIDE software (p-value < 0.05). To the best of our knowledge, CD81 gene editing in these cells has been investigated for the first time in this study using the CRISPR/Cas9 technique.

Conclusions. Taken together, our data show that the CRISPR-Cas9 system can change the genomic sequence in the target area of MDA-MB-231 cells. Along with previous studies, we propose forethought when using T7E1-based quantitative indel estimates, as comparing activities of multiple gRNAs with the T7E1 assay may lead to inaccurate conclusions. Instead, estimating non-homologous end-joining events (NHEJ) by Sanger sequencing and subsequent TIDE analysis is recommended. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 1, 13–23)*

Key words: CRISPR-Cas9; gene editing; CD81 gene; MDA-MB-231 cells; transfection efficiency

Corresponding author: Saeed Kaboli
Department of Medical Biotechnology, School of Medicine,
Zanjan University of Medical Sciences,
and
Cancer Gene Therapy Research Center,
Zanjan University of Medical Sciences,
Zanjan 4513956111, Iran
e-mail: kaboli2017@zums.ac.ir; kaboli2009@gmail.com

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0007
ISSN 0239-8508, e-ISSN 1897-5631

www.journals.viamedica.pl/folia_histochemica_cytobiologica

Introduction

Breast cancer (BC) is the most common cancer and is considered to be one of the leading causes of cancer-related deaths in women worldwide. Despite well-developed strategies for targeting primary tumors, they act poorly when it comes to metastatic malignancies, which are the leading cause of breast cancer-related deaths [1, 2]. The breast tumors are categorized as Basal-like (triple-negative), Luminal A (ER positive-low grade), Luminal B (ER positive-high grade, HER2-positive), which can be encountered more specifically and selectively due to recent progress in molecular biology. Due to the lack of estrogen, progesterone, and HER2 receptors, patients with triple-negative breast cancer cells are highly resistant to conventional therapies [2–4]. Therefore, it is essential to develop more effective treatments based on precise molecular mechanisms to overcome drug resistance. Moreover, several molecular markers in breast cancer can serve as specific targets; for example, CD81 [5].

CD81, a member of the tetraspanin superfamily of proteins, was initially identified as a target of anti-proliferative antibodies by inhibiting B-cell proliferation in lymphoma [6]. Tetraspanins are small membrane proteins that are expressed in all metazoans with 33 members in mammalians, involved in many cellular signaling pathways such as receptor-mediated, adhesion-mediated, and intracellular signaling [7, 8]. The crucial effect of CD81 has been reported in the immune system of CD81-null mice. It was shown that CD81 is not essential for the normal development of T-cells and B-cells [9, 10]; however, it is vital for the proliferation of B-cells as well as the proper functioning of the humoral immune system and the expression of CD19 in the membrane of B lymphocytes [10, 11]. Furthermore, the role of CD81 has been shown in epidermal mesenchymal transition (EMT) and metastasis through regulating TGF-β receptors in melanoma [12]. Additionally, the anti-CD81 antibody showed promising characteristics as a novel immunotherapeutic molecule for B cell lymphomas [13]. Therefore, CD81 may be a potent marker in breast cancer for early therapeutic purposes owing to its multiple functions [14].

Targeted alteration of the genome with designed nucleases is an emerging technology in biomedicine and biotechnology. One of the most recent and promising tools in cancer basic research and therapeutics is attributed to the CRISPR-Cas9 system. This system with its superiorities such as cheapness, high accuracy, specificity, easy design, potency, and versatility, along with techniques such as ZFNs, TALENs, and meganucleases, have been explored as promising tools in human cancer gene therapy [15–18].

Numerous studies in recent years have shown the high efficiency of gene disruption and modification by CRISPR-Cas9 in various targets [18–22]. This crucial potential of the CRISPR-Cas9 system can be exploited for cancer gene and cell therapy. To demonstrate this ability, in the present study, we show for the first time that alteration in specific regions of the CD81 gene can be achieved using the CRISPR-Cas9 technique in the MDA-MB-231 breast cancer cells. To this end, among 10 exons included in the CD81 gene, we focused our attention on exons 3 and 5 since *in silico* analysis estimated that the best sgRNAs can be designed on these exons. Four sgRNAs (PRI to PRIV, Table 1) were designed to target designated sites within exons 3 and 5, and as a result two of the four sgRNAs were conferred 1.5% to 3% editing efficiency.

Materials and methods

Design of sgRNAs and primers. According to the NCBI database, the CD81 gene contains 10 exons. First, a series of sgRNAs were designed within these exon sequences and analyzed using the CRISPR tool available at http://crispr.mit.edu and sgRNA Scorer 2.0 available at https://crispr.med.harvard.edu. Four sgRNAs were chosen based on scores such as minimum off-targets, high efficiency, optimum GC content, low self-complementarity, and also low possibility of secondary structures formation (heterodimer, self-dimer, and hairpin). Then, the required specific primers for amplifying of target regions in the genomic DNA (exon 3 and exon 5), and other primers necessary for screening the sgRNA cloning process and confirming the insertion of sgRNA into the PX458 plasmid were designed. All designed sequences of sgRNAs and primers are listed as (5´-3´) sequences in Table 1.

Cloning of sgRNAs into PX458 plasmid. The designed sgRNAs were synthesized as DNA nucleotide sequences and cloned into digested PX458 plasmid to construct 4 active CRISPR vectors: two of sgRNA for targeting specific regions of exon 3 and another two sgRNAs for targeting specific regions of exon 5 in the CD81 gene in MDA-MB-231 cells. pSpCas9(BB)-2A-GFP (PX458) (plasmid number 48138; Addgene, www.addgene.org) was a gift by Dr. Agnès Bernet. The cloning of sgRNAs into PX458 plasmids was performed following Ran *et al.* protocol [23].

The digestion and cloning steps are discussed in detail below. The PX458 plasmid was digested with BbsI for 16 h at 37°C, followed by heat inactivation for 20 min at 65°C. The digested plasmid was gel-purified using the EZ-10 Spin Column DNA Gel Extraction Kit, according to the

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0007
ISSN 0239-8508, e-ISSN 1897-5631

Table 1. Designed sequences of sgRNAs and primers

Characteristics and sequence of designed sgRNAs						
Name	**Forward sequence (5'-3')**	**Reverse sequence (5'-3')**	**Exon**	**Efficiency**	**Strand**	**Genomic location**
PRI	caccGGTGAAGACCTTCCACGAGA	aaacTCTCGTGGAAGGTCTTCACC	5	64	+	Chr11:2395499
PRII	caccgTGATGACGCCAACAACGCCA	aaacTGGCGTTGTTGGCGTCATCAc	5	69	+	Chr11:2395472
PRIII	caccgTGACAGCGCCCACAGCGATG	aaacCATCGCTGTGGGCGCTGTCAc	3	57	-	Chr11:2394105
PRIV	caccGGCGCTGTCATGATGTTCGT	aaacACGAACATC,ATGACAGCGCC	3	50	+	Chr11:2394118
Genomic DNA primers						
EXON NUMBER	**Forward primer (5'.3')**	**Tm (°C)**	**Reverse primer (5'.3')**		**Tm**	**Product size**
3	GGTAACTGAGGCACAGAAAACTCAC	62	ACCTCCCCCAGAATTCTAGGCT		62	404
5	TCCTCTGGGGTCTAGCCTCGA	63.5	CCACTAGGTTCCCACCCGAGG		64	403
List of primers designed for plasmid PX458						
Name	**Forward Sequence**	**Tm**	**Reverse Sequence**		**Tm**	
Ligation	GAGGGCCTATTTCCCATGATT	64	GGTACCCGTTACATAACTTACGG		65	
Name	**Sequence**				**Tm**	
sgRNA1 ligation screening	AAACACCGGTGAAGACCTTC				65	
sgRNA2 ligation screening	GTGATGACGCCAACAACGC				68	
sgRNA3 ligation screening	AAACACCGTGACAGCGC				67	
sgRNA4 ligation screening	GCGCTGTCATGATGTTCGT				66	

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0007
ISSN 0239-8508, e-ISSN 1897-5631

Table 2. Sequences of designed primers for amplification of target regions in DNA genomic

Exon position	Forward primer (5'.3')	Tm	Reverse primer (5'.3')	Tm	Product size
3	GGTAACTGAGGCACAGAAAACTCAC	62	ACCTCCCCCAGAATTCTAGGCT	62	404
5	TCCTCTGGGGTCTAGCCTCGA	63.5	CCACTAGGTTCCCACCCGAGG	64	403

manufacturer's recommendations (BioBasic Inc, # BS353, Markham, ON, CA). The sense and antisense oligonucleotides (oligos) of each sgRNA were diluted at 100 μM in distilled water. To phosphorylate and anneal the oligos, 1.5 μl of each oligo was mixed with T4 ligation buffer and T4 polynucleotide kinase (T4 PNK) (New England Biolabs, Ipswich, MA, USA) to a final volume of 50 μl and incubated for 30 min at 37°C (phosphorylation), followed by 5 min at 95°C and then ramping down the temperature to room temperature (RT) at –1°C/min (annealing). The annealed and phosphorylated oligos were diluted at 1:10 in distilled water. Ligation reactions for each sgRNA were performed by mixing 100 ng/μl of the digested and purified PX458 plasmid with 2 μl of the diluted phosphorylated and annealed oligos, T4 ligation buffer, and T4 ligase in a final volume of 20 μl. Ligation was carried out at 22°C for 1 hr. The bacterial transformation was performed by mixing 5 μl of the ligation reaction with 50 μl of ice-cold chemically competent *E. coli* stbl3 bacteria. The mixture was incubated on ice for 30 min, followed by a heat shock at 42°C for 30 s and a subsequent incubation on ice for 2 min. The transformation reaction was plated on LB plates containing 50 μg/ml ampicillin, and plates were incubated overnight at 37°C. Colonies were checked for correct insertion of the sgRNA by colony PCR followed by sequencing.

Colony PCR screening and plasmid sequencing. Two bacterial colonies per plate were mixed in PCR tubes with distilled H_2O, PCR Master Mix (Bio Basic), and specific primers (Table 1). PCR was performed at an annealing temperature of 64.9°C for 35 cycles. Positive colonies (with sgRNA insertion) displayed 200 bp PCR amplicon, whereas negative colonies showed no amplicon. Two colonies were picked from each LB plate and inoculated into a 5 ml culture of LB medium supplemented with 50 μg/ml ampicillin. The cultures were grown overnight at 37°C. Plasmid DNA from the bacterial culture was extracted using the EZ-10 spin column plasmid DNA miniprep kit (BioBasic Inc.). Correct insertion of the sgRNA into the PX458 plasmid was verified by sequencing.

Genomic DNA sequencing. Genomic DNA was extracted from the MDA-MB-231 cell using Takapouzist DNA extraction kit, and the concentration of extracted DNA was measured with Thermo Fisher Scientific (Waltham, MA, USA) NanoDrop spectrophotometers. PCR primers for two exons (Table 2) were synthesized to amplify a 400-bp region. The PCR products were loaded on a 1% agarose gel, and the bands were purified using an EZ-10 Spin Column DNA Gel Extraction Kit. The concentration of 30 ng/μl purified PCR products was sent for Sanger sequencing of the 400-bp fragment. Raw sequences were prepared by SnapGene® software (https://www.snapgene.com/) and subsequently analyzed using TIDE online software (https://tide.nki.nl/#about).

Cell culture. The MDA-MB-231 breast cancer cell line was purchased from the Pasteur Institute of Iran (Tehran, Iran). The MDA-MB-231 cell line is the triple-negative breast cancer cell, as it lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) expression. In this work, The MDA-MB-231 cell line was cultured in high glucose (4500 mg/l of glucose) DMEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin (Sigma-Aldrich).

Transfection. MDA-MB-231 cells were cultured in 10% FBS enriched DMEM. In the present study, the liposomal method using DNAfectamine (BioBasic Inc.) was used to deliver recombinant plasmids containing sgRNA. DNAfectamine (DNAF) is a substance consisting of 4 identical formulations of polycations and liposomes that ensure high transfection efficiency and low cytotoxicity in eukaryotic cell lines. Different concentrations of plasmid (0.2 μg/μl, 0.4 μg/μl, 1 μg/μl, 2 μg/μl) and amounts of DNAF (2 μl, 3 μl, 4 μl, 5 μl) were separately dissolved in 25 μl serum- and antibiotics-free high glucose DMEM, followed by 5 min incubation at RT. After that, indicated concentrations of plasmids and DNAFs were combined and prepared plasmid-DNAF complexes. Then, they were added into 24-well culture plates, cultured with MDA-MB-231 cells at 70–90% of cell confluency. Finally, the delivery of plasmid was visually evaluated by fluorescence microscopy and transfection efficiency was quantitatively analyzed *via* flow cytometry. Briefly, 72 h after transfection, transfected cells were collected by trypsinization, washed in ice-cold PBS, and subsequently were re-suspended in the 1× PBS to a concentration of 1×10^6 cells and then applied to flow cytometer BD FACS Calibur (BD Biosciences, San Jose, CA, USA).

Cell viability assay. The MTT assay (Sigma-Aldrich) was used to investigate the cell viability after transfection. MDA-MB-231 cells, 15,000 per well, were seeded in a 96-well plate). After 24 h of incubation at 37°C, the transfection

10.5603/FHC.a2022.0007
ISSN 0239-8508, e-ISSN 1897-5631

process was performed with different concentrations of plasmid PX458 (100 ng/μl and 500 ng/μl) and different amounts of DNAfectamine (2 μl and 1 μl). After 24 h of incubation at 37°C, 20 μl of MTT solution (5 mg/ml) was added to each well, followed by incubation for 3–4 h. Next, cells were exposed to 100 μL dimethyl sulfoxide (DMSO) for about 10 min, and the resulting solution was analyzed at 570 nm using a multi-mode scanning spectrophotometer reader (Thermo Fisher Scientific™ Varioskan™ LUX multimode).

CD81 gene editing validation. The efficiency of gene editing was evaluated at different levels. T7 endonuclease 1 (T7E1) mismatch detection assay was used to evaluate the editing efficiency of different sgRNAs [24]. Moreover, Sanger sequencing was performed following transfection of recombinant plasmids and plasmid PX458 (as control) through PCR reactions of exons 3 and 5. For more detailed investigation, TIDE online software (https://tide.nki.nl/#about) was used to examine the editing efficiency of different sgRNAs.

T7E1 mismatch cleavage assay. T7E1 is an enzyme that recognizes and cleaves mismatches in heteroduplex DNA sequences. This mismatch cleavage assay is a simple, cost-effective, and easy-to-interpret technique for detecting mutations or alterations in the targeted region of genomic DNA to validate CRISPR-based genome editing. To validate genome editing based on the CRISPR system, following transfection, genomic DNA was extracted from MDA-MB-231 cells and then the targeted regions in exon 3 and exon 5 were amplified by PCR. Finally, the amplified products were treated with the T7E1 enzyme to make cleavage in the altered region in the target area.

Statistical analysis. Graphpad Prism 9.0 (Graphpad Software, San Diego, CA) and Microsoft Excel (version 16.37; Microsoft Corp., Redmond, WA, USA) were used for all statistical analyses. At least three independent experiments were performed. All values were presented as mean ± standard deviation (SD). Also, for multiple comparisons, a one-way analysis of variance (ANOVA) was used among three or more groups.

Results

CD81 gene amplification

As described in Materials and Methods, exon 3 and exon 5 of the CD81 gene were amplified using specific primers to sequence the target regions for designed sgRNAs. To ensure the correct sequence of exon 3 and exon 5 of the CD81 gene and to investigate the absence of any mutations, deletions, or additions in the target sequences retrieved from https://www.ncbi.nlm.nih.gov/, PCR products were sequenced and the results are shown in Fig. 1.

Use of ligation to clone sgRNAs into PX458 plasmid

We used 4 sgRNAs targeting the exon 3 and exon 5 regions of the CD81 gene. Each sgRNA was cloned into the PX458 plasmid. Following the recombinant PX458-sgRNA plasmid transfer into Stbl3 bacterial cells, the single proliferating colony cells from the ampicillin agar plate were subcultured. Using PCR, clones were examined for sgRNA sequences existence and electrophoresed on the agarose gel. Finally, the ligation of designed sgRNAs in the PX458 plasmid was verified by sequencing (Fig. 2).

Verification of MDA-MB-231 cells' transfection

The prepared plasmids (PRI, PRII, PRIII, and PRIV) were delivered by DNAfectamine into MDA-MB-231 cells. Cellular uptake and transfection efficiency was evaluated using fluorescence microscopy. As shown in Fig. 3, the emission of fluorescent green color due to GFP reporter gene expression indicates the successful entry of PX458 plasmids into MDA-MB-231 cells.

Cell viability assay

Since the survival of target cells has particular importance in genome engineering, the carrier-CRISPR system complex must have the least toxic effect on target cells. MDA-MB-231 cells were transfected with different concentrations of plasmid and DNAfectamine and analyzed for cell viability. As shown in Fig. 4 (panel A), the results showed that the selected concentration indicated with asterisks (*), has approximately similar toxicity with other ones, but with improved transfection efficacy (panel B).

Transfection efficiency

The transfection efficiency of plasmid-DNAfectamine complexes was quantitatively analyzed by flow cytometry. Flow cytometry analyses showed that among the selected concentration (panel A in Fig. 4) the considered final concentration (*i.e.*, Fig. 4B–1) is associated with the highest efficiency, which is about 27%.

Gene editing efficiency

After performing cellular DNA transfection, the genomic DNA of the transfected cells was extracted, and the selection regions were amplified and purified, followed by the T7E1 assay using the PCR technique. Finally, to investigate the presence of any editing in the desired genomic regions, sequencing was performed and analyzed using TIDE software. The results showed that delivering PRII and PRIII plasmids into MDA-MB-231 cells caused changes in the target regions of the CD81 gene (Fig. 5). According to the TIDE results for PRII, in 3.5% of the total cell population, one base, which is probably of the

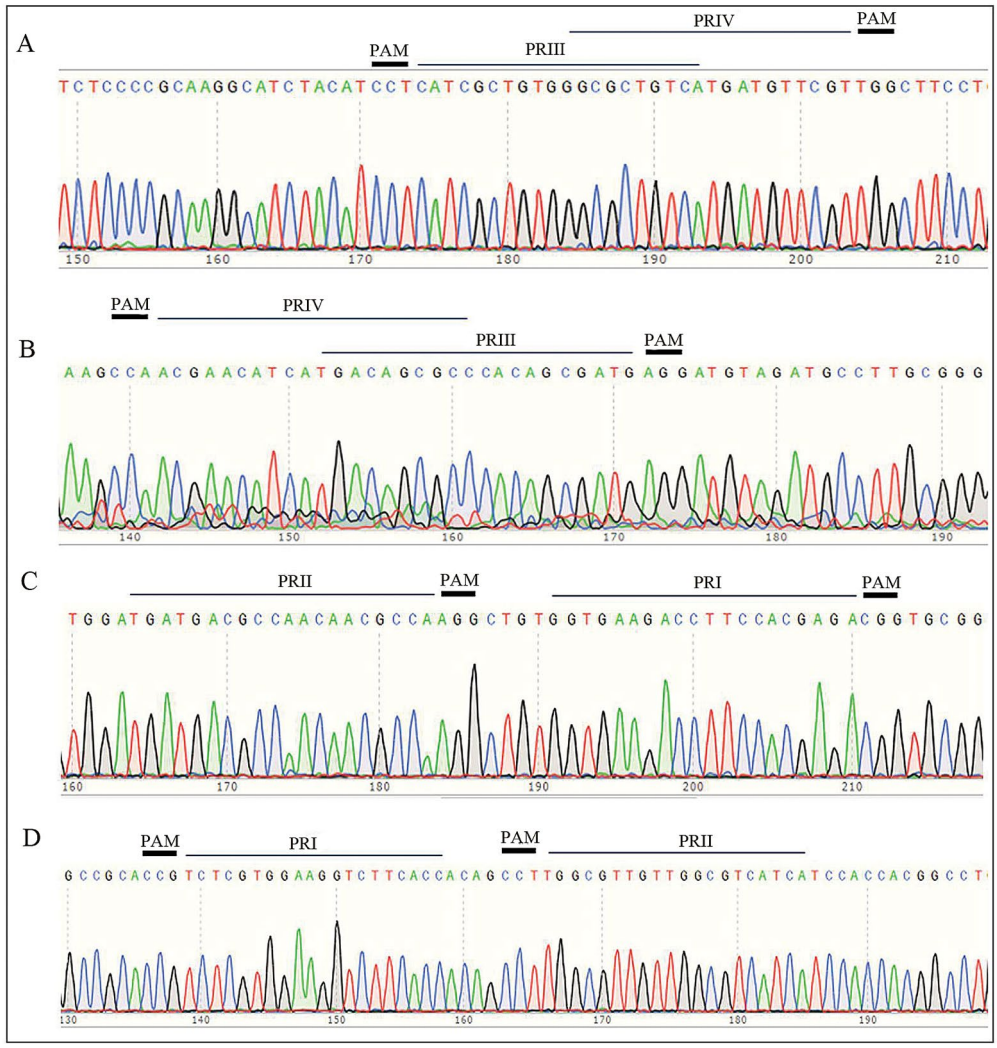

Figure 1. Sequencing of target regions in wild-type genomic DNA of MAD-MB-231 cells (non-transfected cells). **A.** Exon 3 region sequencing with the forward primer. **B.** Exon 3 region sequencing with reverse primer (location of target regions are shown as PRIII, PRIV). **C.** Exon 5 region sequencing with the forward primer. **D.** Exon 5 region sequencing with reverse primer (location of target regions are shown as PRI, PRII).

adenine type, was added to the sequence, and nine bases were added in the target area in 2.6% of the total cell population. However, For PRIII, one base was removed in 1.5% of all cells.

Discussion

The capability of genome editing tools in the acquisition of precise genetic alterations in various organisms is one of the goals that researchers in molecular biology and genetics have been pursuing for years [25]. The advent of the CRISPR method provided a revolution in achieving such changes both in terms of costs and time effectiveness. The CRISPR-Cas9 system, among others, may be used to explore the mechanisms behind the occurrence, development, and metastasis of malignant tumors [26, 27].

To investigate CD81 gene role and function in some malignancies, it had been knocked out by the CRISPR-Cas9 system in several cell lines. Quagliano *et al.* harnessed pSpCas9 (BB)-2A-GFP (PX458) to clone guide sequence (GGCGCTGTCATGATGT-TCGT) targeting exon 3 of CD81 [28]. They successfully knocked out CD81 in precursor leukemia cell lines Nalm6 and Sup-B15. The efficacy characteristic of this guide sequence in CHOPCHOP online software [29] was reported to be 51.71%. In another study, Mizoshiri *et al*. attempted to knock out the CD81 gene in osteosarcoma cell line 143B [30]. They used pLentiCRISPR v2 and ligated twenty bases of the target sequence (TGAGGTGGCCGCCGGCATCT) into it. The efficacy of this sgRNA in CHOPCHOP software was determined to be about 32.56%. However, in our study, the PRII targeting sequence that showed

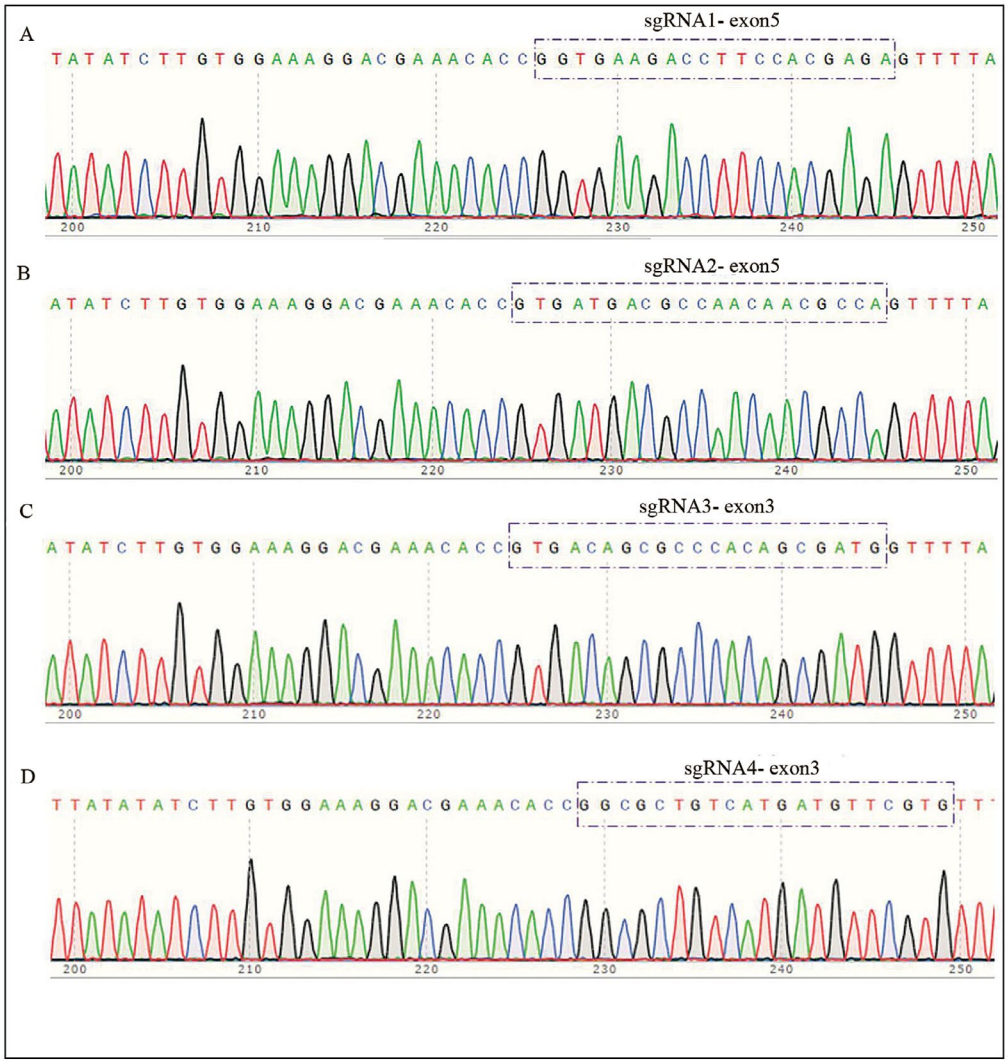

Figure 2. Verifying ligation of designed sgRNAs into PX458 plasmid. Sequencing of regions containing **A**. sgRNA1; **B.** sgRNA2; **C.** sgRNA3; **D.** sgRNA4 in the PX458 plasmid. The sequencing was performed as described in Materials and Methods.

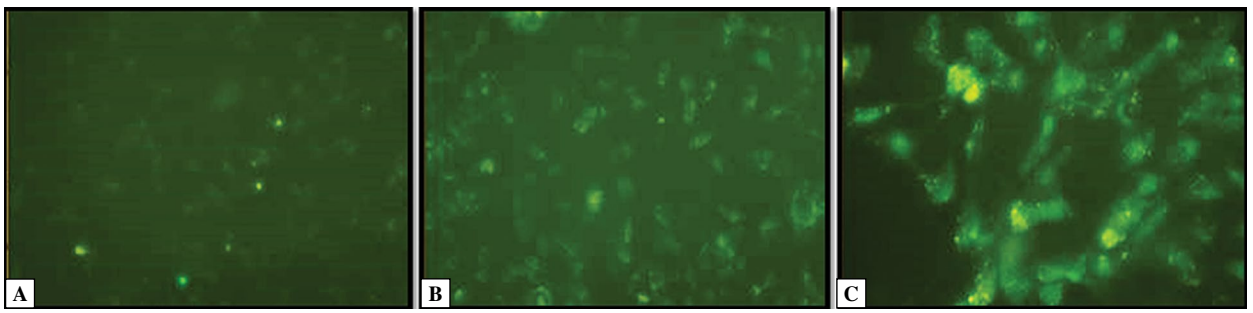

Figure 3. Photomicrographs of EGFP-expressing MDA-MB-231 cells. **A.** Non-transfected cells (negative control). **B** and **C.** Transfected cells. Magnifications: A and B — 20×; C — 40×.

10% of total edition efficacy by TIDE software, had an efficacy of 69% in CHOPCHOP (Table 1). This is where we should notice that in practice several different factors such as transfection efficiency of transfection reagent affect the editing efficiency of a sgRNA, particularly in hard-to-transfect cells. It

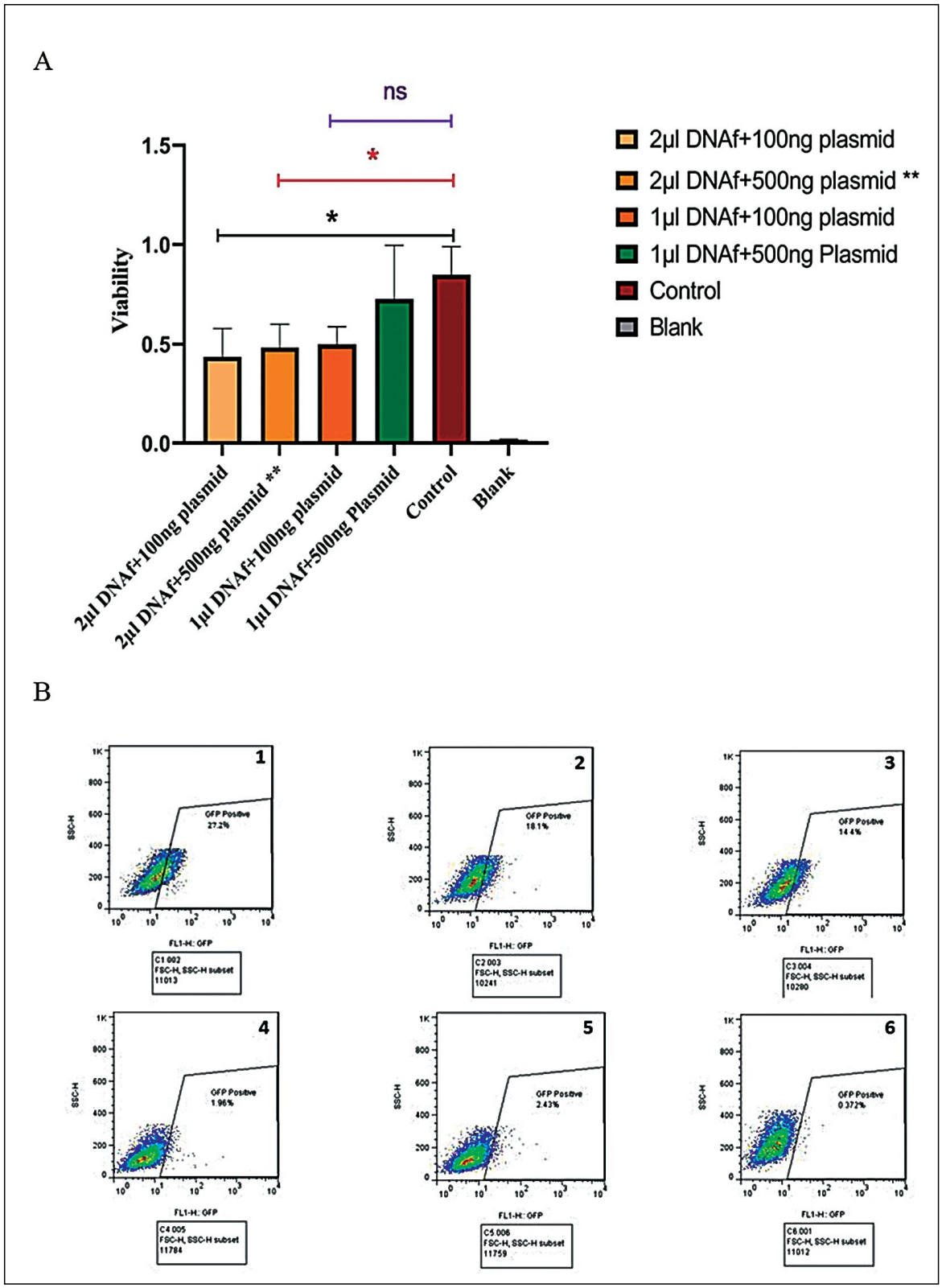

Figure 4. Cell viability assay and analysis of transfection efficiencies of MDA-MB-231 cells transfected with different concentrations of plasmid-DNAfectamine complexes. **A.** Effects transfection solutions on cell viability were determined by MTT assay, control — cells transfected with non-modified PX458 plasmid (*$p < 0.05$). **B.** The results of flow cytometric analysis. Y-axis and X-axis represent side scatter (height of the pulse signal detected) and fluorescence intensity respectively. The transfusion solution contained $x\,\mu$l of DNAfectamine and $y\,\mu$g of PX458 plasmid, respectively. **1**: $x = 8\,\mu$l, $y = 2\,\mu$g; **2**: $x = 4\,\mu$l, $y = 400\,\mu$g; **3**: $x = 4\,\mu$l, $y = 2\,\mu$g; **4**: $x = 4\,\mu$l, $y = 400$ ng; **5**: the transfusion solution contained only 2 μg of PX458 plasmid; **6**: no treatment was performed on the cells.

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0007
ISSN 0239-8508, e-ISSN 1897-5631

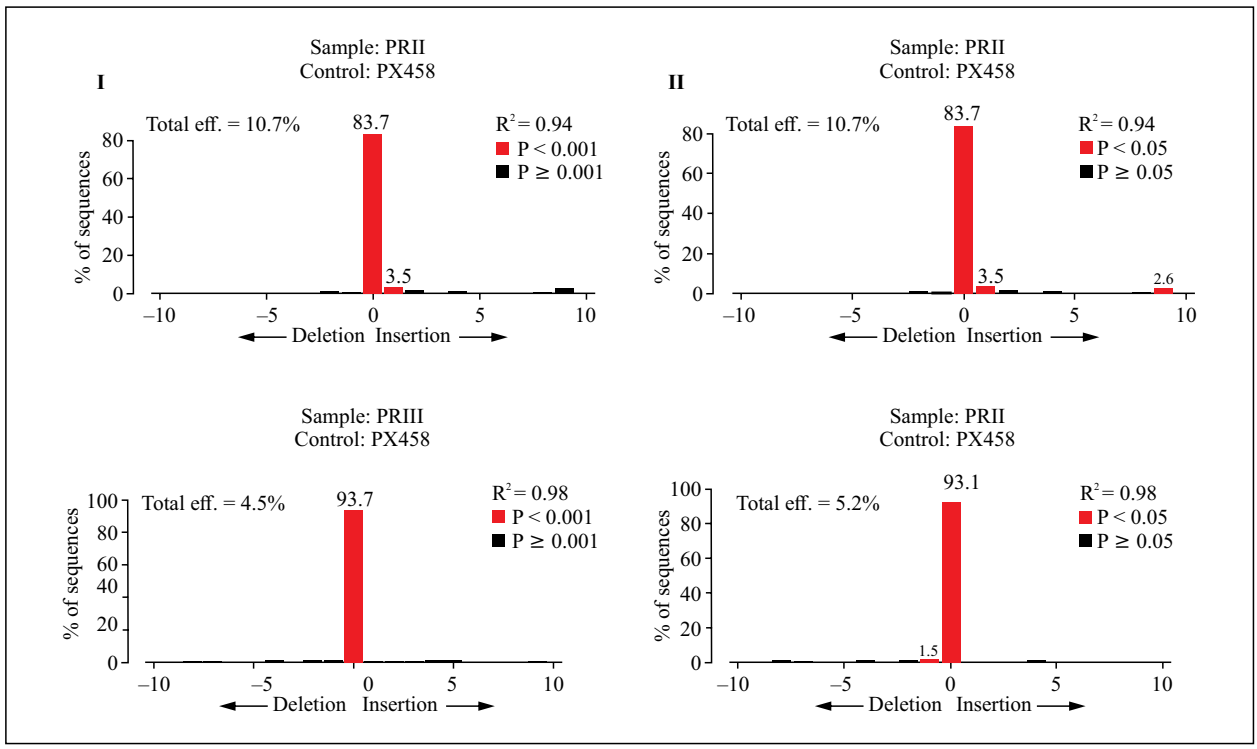

Figure 5. Analysis of targeted gene editing efficiency using TIDE software. I and II represent $p < 0.001$ and $p < 0.05$, respectively.

has been shown that applying purified Cas9 ribonucleoproteins (RNPs) approach for genome editing in human embryonic stem cells in comparison with plasmid-based methods, could improve the editing efficiency up to two-fold [31]. Besides, the efficiency of the CRISPR-Cas9 technique in eukaryotic cells is highly dependent on several factors, including cell transfection efficiency. Therefore, an efficient delivery method for the CRISPR-Cas9 system is necessary, exhibiting high delivery efficiency and minimal toxicity [32]. The commercial transfection agent, DNAfectamine, a type of lipid nanoparticle, was used in this study to set up the CRISPR-Cas9 system-based gene editing and to deliver the plasmid format of the CRISPR-Cas9 system components. Lipid nanoparticles are among the most widely used nanomaterials for nucleic acid delivery and have been evaluated in clinical trials for RNAi-based therapeutic applications and nucleic-acid-based vaccines encapsulation [33, 34]. Negatively charged nucleic acids have been shown to form complexes with positively charged lipid particles through electrostatic bonds that protect the nucleic acids against intracellular nucleases [35, 36]. Lipid-based transfection agent, DNAfectamine, showed up to 27% transfection efficiency for delivery of PX458 plasmid into the MDA-MB-231 cells in this study.

Brinkman *et al.* reported the development and performance of TIDE online software in 2014 [37]. They showed its ability for estimating low editing efficiencies in pooled cells. More specifically, this study confirmed 1.8% and 0.9% edition efficacies in human retinal pigment epithelium and drosophila KC167 cell lines, respectively. In this study, T7E1 assay and TIDE software were used to analyze the sgRNAs editing efficiency for exon 3 and exon 5 of the CD81 gene. In our study, performing the T7E1 assay for any of the designed sgRNAs did not reveal genomic edition. However, after sequencing and using TIDE software, PRII and PRIII plasmids represented the total editing efficiency rate as 10.7% and 5.2%, respectively. Sentmanat *et al.* reported that determinations of nuclease activity estimated by T7E1 usually do not accurately reflect the activity of CRISPR/Cas9 genome editing in pools of cells [38]. More accurately they showed that NHEJ[1] events below 10% in CRISPR-Cas9 targets could not be reported by T7E1 assay. Finally, they showed that targeted NGS, TIDE, and IDAA assays determine approximately the same editing efficiencies for pools of cells.

In summary, although the TIDE analysis revealed a small amount of editing in the target areas of the

[1]The authors declare that there is no conflict of interest.

CD81 gene in the current study, it can be related to the low efficiency of the cell transfection method employed. Only 27% of transfected cells successfully received the PX458 plasmid, which is a plausible reason for the low efficiency of CD81 gene editing in MDA-MB-231 cells, which varies from 1.5% to 3%. As a result, it seems to be possible that improving cellular uptake with efficient delivery techniques would result in a higher incidence of genome editing for the relevant sgRNAs.

Acknowledgments

This work was supported by the Zanjan University of Medical Sciences (grant number: A-11-1294-2).

Conflict of interests

The authors declare that there is no conflict of interest.

References

1. Bilani N, Zabor EC, Elson L, et al. Breast cancer in the United States: a cross-sectional overview. J Cancer Epidemiol. 2020; 2020: 6387378, doi: 10.1155/2020/6387378, indexed in Pubmed: 33178276.
2. Al-Mahmood S, Sapiezynski J, Garbuzenko OB, et al. Metastatic and triple-negative breast cancer: challenges and treatment options. Drug Deliv Transl Res. 2018; 8(5): 1483–1507, doi: 10.1007/s13346-018-0551-3, indexed in Pubmed: 29978332.
3. Tsang J, Tse G. Molecular classification of breast cancer. Adv Anat Pathol. 2019; 27(1): 27–35, doi: 10.1097/pap.0000000000000232, indexed in Pubmed: 31045583.
4. Dai X, Cheng H, Bai Z, et al. Breast cancer cell line classification and its relevance with breast tumor subtyping. J Cancer. 2017; 8(16): 3131–3141, doi: 10.7150/jca.18457, indexed in Pubmed: 29158785.
5. Sporikova Z, Koudelakova V, Trojanec R, et al. Genetic markers in triple-negative breast cancer. Clin Breast Cancer. 2018; 18(5): e841–e850, doi: 10.1016/j.clbc.2018.07.023, indexed in Pubmed: 30146351.
6. Oren R, Takahashi S, Doss C, et al. TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. Mol Cell Biol. 1990; 10(8): 4007–4015, doi: 10.1128/mcb.10.8.4007-4015.1990, indexed in Pubmed: 1695320.
7. Termini CM, Gillette JM. Tetraspanins function as regulators of cellular signaling. Front Cell Dev Biol. 2017; 5: 34, doi: 10.3389/fcell.2017.00034, indexed in Pubmed: 28428953.
8. Schaper F, van Spriel AB. Antitumor immunity is controlled by tetraspanin proteins. Front Immunol. 2018; 9: 1185, doi: 10.3389/fimmu.2018.01185, indexed in Pubmed: 29896201.
9. Maecker HT, Levy S. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. J Exp Med. 1997; 185(8): 1505–1510, doi: 10.1084/jem.185.8.1505, indexed in Pubmed: 9126932.
10. Miyazaki T, Müller U, Campbell KS. Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81. EMBO J. 1997; 16(14): 4217–4225, doi: 10.1093/emboj/16.14.4217, indexed in Pubmed: 9250665.
11. Vences-Catalán F, Kuo CC, Rajapaksa R, et al. CD81 is a novel immunotherapeutic target for B cell lymphoma. J Exp Med. 2019; 216(7): 1497–1508, doi: 10.1084/jem.20190186, indexed in Pubmed: 31123084.
12. Wang HX, Sharma C, Knoblich K, et al. EWI-2 negatively regulates TGF-β signaling leading to altered melanoma growth and metastasis. Cell Res. 2015; 25(3): 370–385, doi: 10.1038/cr.2015.17, indexed in Pubmed: 25656846.
13. Küppers R. CD81 as target for B cell lymphomas. J Exp Med. 2019; 216(7): 1469–1470, doi: 10.1084/jem.20190733, indexed in Pubmed: 31171705.
14. Vences-Catalán F, Duault C, Kuo CC, et al. CD81 as a tumor target. Biochem Soc Trans. 2017; 45(2): 531–535, doi: 10.1042/BST20160478, indexed in Pubmed: 28408492.
15. Jamehdor S, Zaboli KA, Naserian S, et al. An overview of applications of CRISPR-Cas technologies in biomedical engineering. Folia Histochem Cytobiol. 2020; 58(3): 163–173, doi: 10.5603/FHC.a2020.0023, indexed in Pubmed: 32978771.
16. Nargesi S, Kaboli S, Thekkiniath J, et al. Recent advances in genome editing tools in medical mycology research. J Fungi (Basel). 2021; 7(4), doi: 10.3390/jof7040257, indexed in Pubmed: 33808382.
17. Ballios BG, Pierce EA, Huckfeldt RM. Gene editing technology: Towards precision medicine in inherited retinal diseases. Semin Ophthalmol. 2021; 36(4): 176–184, doi: 10.1080/08820538.2021.1887903, indexed in Pubmed: 33621144.
18. Das BD, Paudel N. A Review on reliability and validity of CRISPR/Cas9 technology for gene editing. Jordan J Biol Sci. 2021; 14(03): 503–511, doi: 10.54319/jjbs/140316.
19. Hendriks D, Clevers H, Artegiani B. CRISPR-Cas tools and their application in genetic engineering of human stem cells and organoids. Cell Stem Cell. 2020; 27(5): 705–731, doi: 10.1016/j.stem.2020.10.014, indexed in Pubmed: 33157047.
20. Khodadad N, Fani M, Jamehdor S, et al. A knockdown of the herpes simplex virus type-1 gene in all-in-one CRISPR vectors. Folia Histochem Cytobiol. 2020; 58(3): 174–181, doi: 10.5603/FHC.a2020.0020, indexed in Pubmed: 32937678.
21. Zhang Ju, Cui ML, Nie YW, et al. CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus. FEBS J. 2018; 285(15): 2828–2839, doi: 10.1111/febs.14520, indexed in Pubmed: 29802684.
22. Sasano Yu, Nagasawa K, Kaboli S, et al. CRISPR-PCS: a powerful new approach to inducing multiple chromosome splitting in Saccharomyces cerevisiae. Sci Rep. 2016; 6: 30278, doi: 10.1038/srep30278, indexed in Pubmed: 27530680.
23. Ran FA, Hsu PD, Wright J, et al. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013; 8(11): 2281–2308, doi: 10.1038/nprot.2013.143, indexed in Pubmed: 24157548.
24. Babon JJ, McKenzie M, Cotton RGH, et al. The use of resolvases T4 endonuclease VII and T7 endonuclease I in mutation detection. Methods Mol Biol. 2000; 152(1): 187–199, doi: 10.1385/1-59259-068-3:187, indexed in Pubmed: 10957979.
25. Jiang C, Meng L, Yang B, et al. Application of CRISPR/Cas9 gene editing technique in the study of cancer treatment. Clin Genet. 2020; 97(1): 73–88, doi: 10.1111/cge.13589 , indexed in Pubmed: 31231788.
26. Liu Y, Zeng Y, Liu Li, et al. Synthesizing AND gate genetic circuits based on CRISPR-Cas9 for identification of bladder cancer cells. Nat Commun. 2014; 5: 5393, doi: 10.1038/ncomms6393, indexed in Pubmed: 25373919.
27. García-Tuñón I, Alonso-Pérez V, Vuelta E, et al. Splice donor site sgRNAs enhance CRISPR/Cas9-mediated knockout efficiency. PLoS One. 2019; 14(5): e0216674, doi: 10.1371/journal.pone.0216674, indexed in Pubmed: 31071190.
28. Quagliano A, Gopalakrishnapillai A, Kolb EA, et al. CD81 knockout promotes chemosensitivity and disrupts in vivo homing and engraftment in acute lymphoblastic leukemia.

Blood Adv. 2020; 4(18): 4393–4405, doi: 10.1182/bloodadvances.2020001592, indexed in Pubmed: 32926125.
29. Montague TG, Cruz JM, Gagnon JA, et al. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res. 2014; 42(Web Server issue): W401–W407, doi: 10.1093/nar/gku410, indexed in Pubmed: 24861617.
30. Mizoshiri N, Shirai T, Terauchi R, et al. The tetraspanin CD81 mediates the growth and metastases of human osteosarcoma. Cell Oncol (Dordr). 2019; 42(6): 861–871, doi: 10.1007/s13402-019-00472-w, indexed in Pubmed: 31494861.
31. Kim S, Kim D, Cho SW, et al. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 2014; 24(6): 1012–1019, doi: 10.1101/gr.171322.113, indexed in Pubmed: 24696461.
32. Liu C, Zhang Li, Liu H, et al. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. J Control Release. 2017; 266: 17–26, doi: 10.1016/j.jconrel.2017.09.012, indexed in Pubmed: 28911805.
33. Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, et al. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet. 2014; 383(9911): 60–68, doi: 10.1016/S0140-6736(13)61914-5, indexed in Pubmed: 24094767.
34. Coelho T, Adams D, Silva A, et al. Safety and efficacy of RNAi therapy for transthyretin amyloidosis. N Engl J Med. 2013; 369(9): 819–829, doi: 10.1056/NEJMoa1208760, indexed in Pubmed: 23984729.
35. Mali P, Aach J, Stranges PB, et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol. 2013; 31(9): 833–838, doi: 10.1038/nbt.2675, indexed in Pubmed: 23907171.
36. Cong Le, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013; 339(6121): 819–823, doi: 10.1126/science.1231143, indexed in Pubmed: 23287718.
37. Brinkman EK, Chen T, Amendola M, et al. Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. 2014; 42(22): e168, doi: 10.1093/nar/gku936, indexed in Pubmed: 25300484.
38. Sentmanat MF, Peters ST, Florian CP, et al. A survey of validation strategies for CRISPR-Cas9 editing. Sci Rep. 2018; 8(1): 888, doi: 10.1038/s41598-018-19441-8, indexed in Pubmed: 29343825.

Submitted: 13 October, 2021
Accepted after reviews: 5 February, 2022
Available as AoP: 14 February, 2022