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Bioinformatics design of CRISPR guide RNA for genomic knockout of ABCB1 gene

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Abstract

Background: Over-expression of P-Glycoprotein (Pgp) induces acquired drug resistance. Therefore, targeting Pgp as a dominant efflux transporter involved in emergence of multidrug resistance (MDR) has become a major strategy for reversibility of sensitivity to chemotherapy.
Objectives: The aim of this study was to design sgRNAs targeting ABCB1 in order to knockout and inhibit the expression of Pgp in Adriamycin resistant (A2780/ADR) ovarian cancer cell line.
Methods: This study was performed as a bioinformatics and computational research in Qazvin University of Medical Sciences in collaboration with the Isfahan University of Medical Sciences during 2015-2016. All the 28 exons of the ABCB1 gene were separately investigated in terms of single guide RNA (sgRNA) target sites with regards to the highest on-target and lowest off-target activities, using www.deskgen.com website. Three sgRNA sequences were chosen and synthesized by the GeneCopoeia company. All the plasmids were validated after extraction using BamH1 and EcoR1 restriction enzymes.
Results: Sequences of the three sgRNAs were selected close to the start codon (ATG) in order to maximize the possibility of exons 4 and 5 knockout. Digested pCRISPR-CG01, using BamH1 and EcoR1, was electrophorized on 1.5% agarose gel. Detection of the two 330bp and 10100bp fragments on the gel confirmed the integrity of the plasmid and success of the restriction enzyme digestion.
Conclusions: The vectors containing the designed sgRNA sequences and CRISPR associated protein (Cas9) can inhibit Pgp gene expression in cell lines over-expressing this gene, including A2780/ADR.

Keywords: Drug Resistance, P-Glycoprotein, Ovarian Cancer, CRISPR

1. Background

Resistance to chemotherapy is a major issue in treatment of ovarian cancer, so that many tumours that initially respond to treatments, relapse and become resistant to several anticancer agents with various structures and mechanisms of action. This phenomenon is known as multidrug resistance (MDR) (1,2). Drug resistance can be caused by various mechanisms, including changes in pharmacokinetics and drug metabolism, changes in the expression or function of the drug target (e.g., up-regulated expression or replication of the gene, increased expression of β-tubulin isotypes and mutations in topoisomerase II), drug degradation in cell organelles, changes in the pathways involved in repairing DNA damage caused by the drug, changes in apoptosis signalling pathways and expression of the proteins that are directly involved in cellular trafficking of the drug (3). One of the main mechanisms of drug resistance is the expression of efflux transporters of the drug in cancer cells, which subsequently reduces the intracellular accumulation of chemotherapy agents that leads required administration of higher doses of the drug (4).

Many of the drug resistance mechanisms are mediated by efflux pumps, such as the members of adenosine triphosphate (ATP)-binding cassette (ABC) transporters superfamily (3). Most of the ABC transporters contain at least two transmembrane and two nucleotide-binding domains and their main function is to efflux a wide range of drugs and xenobiotics out of the cell (5,6). Approximately, 48 isoforms of ABC transporters have been identified which are categorized into seven subfamilies (ABCA-ABCG), based on the homology in their nucleotide-binding domain (6,7). Pgp is one of the most recognized human efflux transporter and is known to be the key mediator of resistance to a wide range of anticancer

drugs (8). The most common ABC transporter involved in MDR is Pgp which has several broad features and identifies hundreds of compounds as small as 300 to 4000 Da. Pgp is therefore considered as an important drug efflux transporter in cancer studies (9). ABCB1, the Pgp encoding gene, is located on chromosome 7, at q21, and consists of 28 exons which encode a protein of 1280 amino acids (6). Pgp consists of 12 cytosolic hydrophobic membrane domains and 2 nucleotide-binding domains. The protein is arranged in two homogeneous halves, which are joined by a flexible linker region. Each half contains 6 cytosolic membrane domains and one cytosolic ATP-binding domain. The cytosolic domains are responsible for determining the properties of the substrate, while the nucleotide-binding domains are responsible for binding to ATP and hydrolysis, leading to the transfer of the substrates (10,11).

Since a slight up-regulation in Pgp expression in cancer cells leads to complete drug resistance, Pgp is known as the main efflux transporter which is involved in the development of MDR (12). Accordingly, inhibiting Pgp activity has been became a major strategy for reversing sensitivity to chemotherapy over the past 20 years (12,13). Inhibitory strategies include inhibiting drug binding to Pgp, inhibiting the activity of Pgp-ATPase, interference with its surrounding environment (the lipidic bilayer) (14) and knockdown of the Pgp gene expression using various techniques, such as microRNAs and RNAi (15).

In the past decade, a new approach has emerged that enables researchers to manipulate virtually any gene in a wide variety of cells and organisms. This major technology, usually called the genome-editing, is based on the application of engineered chimeric nucleases that consists of sequence-specific DNA-binding domains. Among these chimeric nucleases are Zinc-Finger Nucleases (ZFN) and Transcription Activator Like Effector Nucleases (TALENs) that allow inducing efficient and accurate genetic changes through the induction of a double-strand break (DSB) at a specific target sequence followed by stimulation of cellular DNA repair pathways, including error-prone non-homologous end joining (NHEJ) and the homologous recombination (HR). CRISPR/Cas systems are the most recently discovered member of this set of dedicated target-specific genomics engineering tools. Although each of these three methods of gene modification has its own advantages and disadvantages, the rapidity and facility of applying CRISPR/Cas systems for manipulating any genes in various types of cells and organisms makes it a novel and excellent genome engineering tool with an extraordinary performance (16,17)sequence-specific DNA-binding modules linked to a nonspecific DNA cleavage domain. ZFNs and TALENs enable a broad range of genetic modifications by inducing DNA double-strand breaks that stimulate error-prone nonhomologous end joining or homology-directed repair at specific genomic locations. Here, we review achievements made possible by site-specific nuclease technologies and discuss applications of these reagents for genetic analysis and manipulation. In addition, we highlight the therapeutic potential of ZFNs and TALENs and discuss future prospects for the field, including the emergence of clustered regulatory interspaced short palindromic repeat (CRISPR.

The aim of the present study was to use bioinformatic tools to design single guide RNAs (sgRNAs) targeting the ABCB1 gene encoding Pgp, using the online DeskGen reference (https://www.deskgen.com), in order to knockout and inhibit the expression of the target gene.

2. Methods

2.1 Study Design and Bioinformatics Tools

The transcript sequence of the ABCB1-202 gene with the ID number of ENST00000622132.4 was extracted from the Ensemble database. The free and easy to use https://www.deskgen.com web site was used to design the knockout experiment considering the fact that the ultimate aim of this study was to knockout ABCB1 gene (Fig. 1.A).

The name of the intended gene, which was ABCB1, was typed in the box appeared on the new page and Homo sapiens was chosen from the drop-down menu (Fig. 1.B). On the new page, maps of all the transcripts of the desired gene, as in the Ensemble, were displayed in their original direction (Forward or Reverse). The promoter regions, the exons (including the protein coding regions and the 3UTR & 5UTR regions) as well as the stop codons were shown in distinct colours. To make it more convenient, the intended variant number was selected from the relevant box and other variants became hidden for easier investigation of the exons of the target gene. The variant 202 was selected in the present study (Fig. 1.C). At this database, the spCas9 nuclease and 20 bp as the length of the sgRNA were set as the default. The nuclease can be changed using the "Option" toolbar and selecting "Set Nuclease".

The black circles on both forward and reverse sequences with different numbers represent the positions of the PAM regions and the corresponding strand, namely, the hybridation position with the sgRNA and it is, in fact, the cutting sites (Fig. 1.D). The numbers inside these circles indicate the on-target activity score of the desired sgRNA. This score predicts the activity of sgRNA and subsequently the probability of cutting the double-strand DNA by the CRISPR nuclease. Through this scoring system, it is possible to distinguish the powerful sgRNAs from weaker ones. The scoring range is from zero to 100. Therefore, it is ideal to select the sgRNAs with the highest score (closest to 100). In this database, sgRNAs with the scores higher than 60 are considered as to be in a good threshold for the proper functioning of the CRISPR system. However, if the sgRNA score out of this range, the threshold number can be reduced. Furthermore, it is possible to enhance the targeting efficiency by choosing the preserved exons among all the variants of the in-

tended gene. This can be useful for the complete knockout of the cells expressing multiple variants of a gene. In this study, all 28 exons of ABCB1 gene were separately examined in order to find the best regions containing the PAM sequence. Moreover, the threshold for appropriate sgRNA sequences was considered to be 50. By selecting each of the exons, separately, and choosing the desired threshold, the list of all the disposed sgRNA sequences in the intended exon is determined. The desired information, including the exact location of the cutting site by nuclease, the GC content (ideally 30-70%) and the on-target activity score are determined by selecting any of the sequences (Fig. 2. E). Afterward, by choosing the "Find off-targets" option, the off-target activity score of the selected sgRNA, as well as the information about the potential regions for off-target activity are illustrated (Fig. 1.F). Therefore, all the exons were individually selected to find the regions containing PAM sequences, with a threshold over 50 and less off-target activity. The sites with a single base-pair mutation (1bp mismatches) or more than one off-target matches with a two base-pair mutation (2bp mismatches) were discarded.

The next step was to send the list of all the regions containing PAM sequences and the selected sgRNAs to the Chinese GeneCopoeia company for further analysis and selection of the most effective sgRNA. Finally, after complementary analyzes using other algorithms, 3 sgRNA sequences were selected close to the start codon (ATG), in order to maximize the probability of knockout of the both exons, 4 and 5. The selected sgRNA sequences were then cloned into the intended plasmid along with the sequence of the Cas9 gene, promoter region, mCherry reporter gene and other required sequences. The final product was synthesized and delivered in lyophilized form by the GeneCopoeia Company.

2.2 Plasmid Propagation

According to the manufacturer's instructions, the vectors were dissolved in 300-400µl of deionized water and transformed into competent TOP10 cells using calcium chloride approach, mediated by thermal shock. Clones containing pCRISPR-CG01 vectors were cultured overnight in a liquid LB culture medium supplemented with ampicillin, at 37 °C. Plasmid purification was performed using the GeNet Bio Plasmid Extraction Kit (GenetBioInc, Korea).

2.3 Digestion Analysis

The extracted plasmid was electrophorised on 1% agarose gel in order to confirm the success of the purification process. To ensure the accuracy and integrity of the extracted plasmids, *BamH1* and *EcoR1* restriction enzymes were applied, using the common buffer Tango, to perform the enzymatic digestion at 37 ° C for 4 hours. Products of the digestion procedure were finally loaded on a 1.5% agarose gel.

3. Results

The steps of selecting the target gene for designing the appropriate sgRNAs are presented in Figure 1.

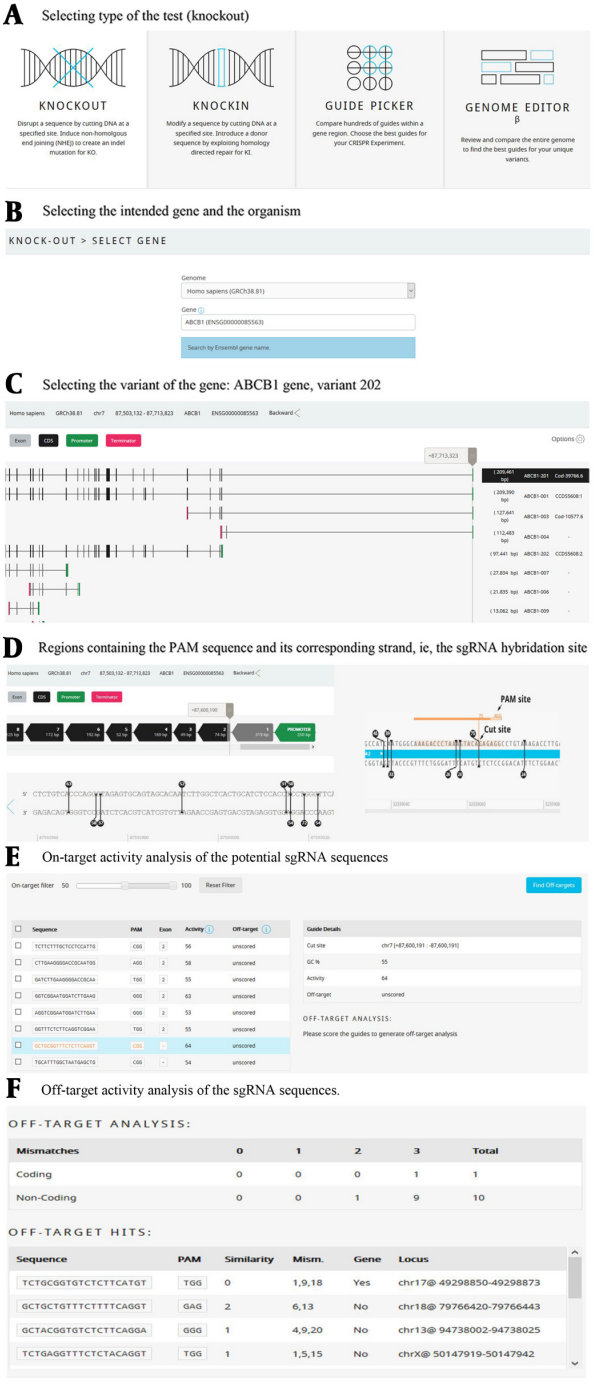

Figure 1. Example of outputs of the DeskGen Web site for identifying sgRNA target locations. A) Selecting type of the test (knockout). B) Selecting the intended gene and the organism. C) Selecting the variant of the gene: ABCB1 gene, variant 202. D) regions containing the PAM sequence and its corresponding strand, ie, the sgRNA hybridation site. E) On target activity analysis of the potential sgRNA sequences. F) Off-target activity analysis of the sgRNA sequences.

The intended designed construct was provided by the Chinese GeneCopoeia company, cloned into the pCRISPR-CG01 plasmid with a size of 10442 bp (Fig. 2). The sgRNAs target sequences (exons 4 and 5), along with the PAM region, are presented in Table 1.

Considering that the sites of action of the *EcoR1* and *BamH1* restriction enzymes were respectively located at base 10003 and 10335 of the plasmid sequence, results of electrophoresising digested pCRISPR-CG01 vector by *BamH1* and *EcoR1* enzymes loaded on a 1.5% agarose gel along with a 1 Kb DNA marker and detecting two bands, with approximate length of 330 bp and 10100 bp, confirmed the integrity of the plasmid and the digestion process (Fig. 3). Finally, concentration of the confirmed plasmids was determined in order to be used for transfection of the ovarian epithelial cancer cells to target and knockout the ABCB1 gene which encode the Pgp membrane transporter.

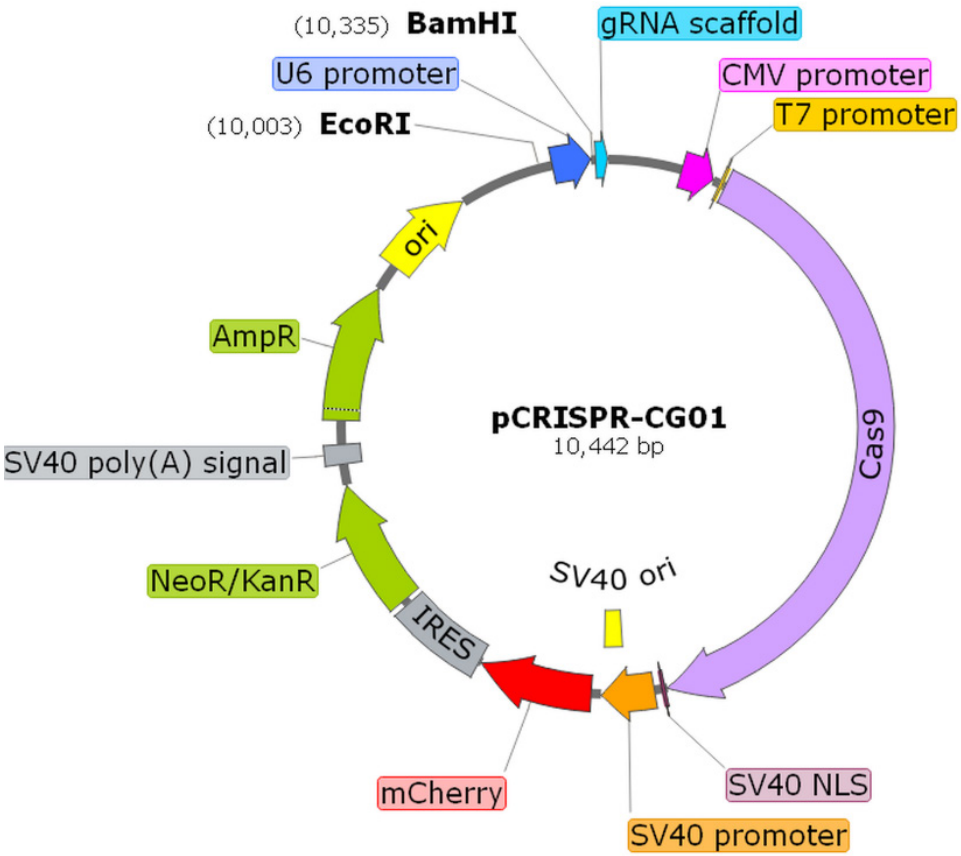

Figure 2. Map of sgRNA clone. The selected sgRNA sequences were cloned into plasmid along with the sequence of the Cas9 gene, promoter region, mCherry reporter gene and other required sequences.

Table 1. The selected sgRNAs target sequences along with PAM region and off-target activity rate.

Sequence	PAM	Exon	Mismatches 1 bp	Mismatches 2 bp	Mismatches 3 bp	Off-target score
TGACAAGTTGTATATGGTGG	TGG	4	0	0	11	370
CCAAACACCAGCATCATGAG	AGG	4	0	0	22	444
CTAGGTGATATCAATGATAC	AGG	5	0	1	4	313

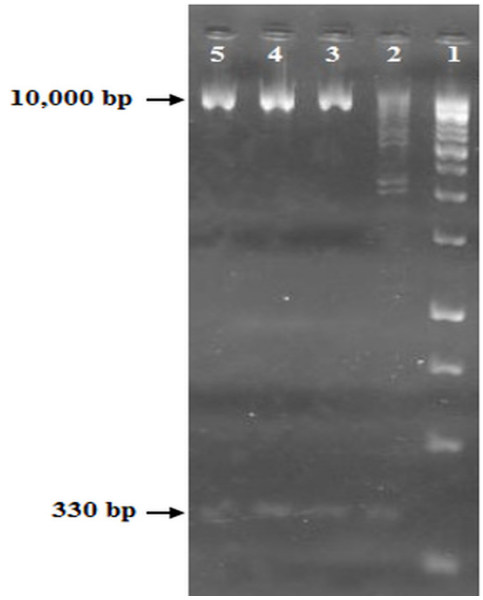

Figure 3. Enzymatic digestion of the pCRISPR- CG01 plasmid. Column 1: 1 Kb DNA marker, Column 2: The pCRISPR GC01 plasmid digestion product with both the BamH1 and EcoR1 enzymes and detection of two bands of 330 and 10100 bp.

4. Discussion

Many human cancer cells express the ABCB1 gene at a level that leads to multi-drug resistance. In fact, based on the analysis of a wide range of cancers, about 50% of the human malignancies over-express ABCB1 gene during the course of treatment, which reduces the impact of chemotherapy and defects the treatment outcomes. Therefore, targeting Pgp has led to the development of Pgp inhibitors that can inhibit the transmission of the substrate and lead to an increase in the intracellular accumulation of the drug (5,18). Over the past three decades, a wide range of ABCB1 inhibitors have been developed; however, none of them have been approved for clinical use. The main disadvantages of these ABCB1 inhibitors are nonspecific toxicity, as well as toxic interactions between the inhibitor and the chemotherapy agents used to treat the patient (19,20). Nonetheless, genetic tools are promising approaches to overcome drug resistance in cancer. In the context of silencing the gene expression after transcription, RNAi is recognized as a leading tool for targeted knockdown of the gene. However, transmission constraints, low stability on blood flow, unwanted and unpredictable off-target effects and ultimately stimulation of the immune system are the main limiting factors of RNAi in terms of therapeutic applications (16,21,22). The most recent genomic engineering tools include protein domains (ZFN or TALE) that bind to specific regions on DNA and are able to manipulate the structure and function of the genome by integrating with a variety of effecter domains. These effecter domains include nucleases, transcriptional activators or repressors, recombinases, transposases, DNA and histone methyltransferases as well as histone acetyltransferases (16). ZF and TALE-based nucleases require detailed and distinct design for any genome site and are, in fact, known as custom-designed nucleases (CDNs). Therefore, their design is more pricey and time consuming compared to the most recent generation of genomic tools, CRISPR systems. The new CRISPR/Cas system, as a potential effective alternative to the ZFN and TALEN tools, is able to induce targeted genetic changes. These sgRNA-based nucleases, in addition to being convenient, inexpensive and quickly designed, have shown more efficiency and flexibility than CDNs and are significant potential subjects in the field of biological and medical studies (16,23). Fortunately, development of new CRISPR/Cas variants, as well as their ability to target multiple genetic locations simultaneously, makes achieving ultimate therapeutic goals with maximum efficiency possible (24). In addition to recent advances in generating more computational resources in the field of genome editing, the simultaneous application of other available "in silico" resources to study genome sequencing is recommended. These resources and algorithms have the potential to provide new aspects of genomic editing tools in terms of bioinformatics design as well as post-genome editing analysis. Although the emergence of computational resources has been rapidly increasing in order to enhance the efficiency of genome editing tools, online resources and accurate computational algorithms are also required for post-editing down-stream analysis as well as total genomic scanning (25).

4.1 Conclusion

In the present study, the sequences of all the 28 exons of the ABCB1 gene were investigated, using the DeskGen computational tool, in order to find the best target sites for the sgRNA. Finally, three sgRNAs, targeting exons 4 and 5, were selected for complete suppression and knockout of Pgp expression in order to evaluate the potential reversibility of sensitivity to chemotherapy agents in a drug-resistant cancer cell line.

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Footnotes

Author's Contribution: All authors helped for designing, analyzing the results and writing the current manuscript.
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