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Research Article

A Systematic Biology Advances on sgRNA Design to Develop an Immunogenic Drug against Cancer Using CRISPR

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ABSTRACT

In the field of genome editing, CRISPR/Cas9 is more suitable for clinical therapeutic applications, especially in the treatment of cancer. Here we wanted to design sgRNA that targets specific genes of helper T cells and cytotoxic T cells to alter them so that they can attack tumor cells. In other words, activating the immune system to treat cancer through the development of an immunogenic living drug. Among the main methods, the sgRNA of the target genes (*PDCD-1*, *CTLA-4* and *NYESO-1*) was designed using the bioinformatics tool Benchling. The designed sgRNA was then cloned *in silico* into a lentivirus-based vector using the SnapGene gene designer. Isolation of an immunogenic drug can be done by transferring designed clones *in vitro* into the host and isolating pseudo-HIV viruses from the host, which consists of designed sgRNAs along with the CRISPR/Cas-9 machinery as a genome surrounded by an HIV viral envelope capable of binding T helper cells and cytotoxic T cells detected by protein-protein docking with PatchDock, followed by significantly improved results by FireDock and visualization in BIOVIA discovery studio. The sgRNAs were successfully engineered with good efficiency and potency to knock out *PDCD-1* and *CTLA-4* along with knock-in of *NYESO-1*. The developed immunogenic live drug has also shown positive results in binding efficiency to both T helper cells and cytotoxic T cells. Overall, this study showed that the key *in silico* results can be helpful in the development of immunogenic live drugs and will serve as a future aspect for *in vitro* treatment of cancer.

Keywords: Genome editing; CRISPR/Cas9; Immunogenic living drug; *PDCD-1*; *CTLA-4*; *NYESO-1*

INTRODUCTION

Genome editing is the use of a set of tools and techniques to manipulate the underlying genetic code or sequence of any organism. These changes can include the addition or deletion of base sequences. In this technology, the human genome sequence can be manipulated precisely to achieve a therapeutic effect on the betterment of human life [1]. Nowadays, the three main and most used types of genomes editing mechanisms include programmable nucleases like ZFNs, TALENs, and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). They follow the DNA repair pathways NHEJ and HDR through DSB on the target [2]. The first technologies (ZFNs and TALENs) now suffer from lower specificity due to their off target side effects [3]. In which recent advances in CRISPR as a genome editing tool become the most power full technology in genetic engineering. CRISPR system with Cas9 protein coding genes is the more valuable, vigorous, and multiplexable genome editing tool among all other types because firstly, it has the function as a single protein effector molecule which does not require other helping Cas proteins unlike in class I types of Cas proteins. Secondly, CRISPR/Cas9 comprises of a nonspecific Cas9 nuclease and a set of programmable sequence specific CRISPR-RNA (crRNA), which can guide Cas9 along with Tra-crRNA(trans-activating CRISPR-RNA), spacer sequences, and PAM (Proto-spacer Adjacent Motif) sequence(5'-NGG-3') to

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cleave DNA and generate double strand breaks at target sites. Thirdly, it has two functional domains-the HNH nuclease domain and RuvC like nuclease domain in the generation of a DSB at the target site. So, among all the types of the CRISPR/Cas system, researchers found the CRISPR/Cas9 is the more powerful whole genome editing tool than others [4].

In the CRISPR/Cas9 mechanism, transcribed pre-Cr RNA of CRISPR/Cas9 first forms RNA duplex with Tra-crRNA as Tra-crRNA is partially complementary to pre-crRNA. This duplex is cleaved by RNase III and an RNA specific ribonuclease, to form a crRNA: Tra-crRNA hybrid known as single-guide RNA (sgRNA) [5]. Due to the specific and unique working mechanism of CRISPR/Cas9, CRISPR based perspectives have become an important tool in designing sgRNA to hit specific targets as the system can be applied to generate gene knockouts in various species by directly introducing specific sgRNA which targets the specific gene into genome, leading to a DNA Double-Strand Break (DSB) at a specified locus. So, designing specific sgRNA enhances the specificity of CRISPR technology which allows complete functional knockout and generates phenotypes more uniformly than other systems such as RNAi [6].

In recent years, cancer immunotherapy made a significant breakthrough using monoclonal antibodies to block the *CTLA-4* and PD-1 immune checkpoints leading to autoimmune side effects. This shows the use of tumour antigens specifically Cancer/Testis Antigens (CTA) into cancer immune therapy can be useful to treat tumours [7]. *NY-ESO-1* or New York oesophageal squamous cell carcinoma 1 is a Cancer-Testis Antigen (CTAs) which shows the most useful and promising criteria among all CTA for cancer immunotherapy as its tumour expression is correlated to elicit spontaneous humoral and cellular immune responses, together with its restricted expression pattern in a wide range of malignancies [8]. In the Immune system, T cells (T-helper cells and cytotoxic T cells) protect the human body from infection by pathogens. It also clears the mutant cells through specific recognition of antigens or immunogens by T Cell Receptors (TCRs). Thus, researchers found that unleashing the inhibition of immune checkpoints such as CTLA-4 and PD-1 along with modification of T cell receptors by genetic engineering of *NY-ESO1* on it, generates genetically engineered T cells which able to boost the antitumor activity of T cells [9].

Lentiviruses (HIV) and retroviruses have the advantage that they can reliably integrate DNA into host cells which can support long term expression, and another is the tolerance capacity for exceptionally large inserts [10]. Which makes the thing easier to think about developing the drug within the virus coat along with surface immunogenic substances so they can only invade by immune cells. Using likewise machinery inside the CRISPR/Cas9 system can be useful to develop a drug that can automatically invade immune cells and deliver the CRISPR/Cas9 into immune cells. The lentivirus based vectors are more helpful because it holds pseudo-HIV regions which allow the vector to express into a host and synthesize a new immunogenic drug which consists of HIV coat to attack immune cells, inside it consists of CRISPR/Cas9 system along with specific sgRNAs as genetic material and the whole pseudoorganism acts as a living immunogenic drug.

This study aimed to design an immunogenic "living drug" for cancer treatment using an *in silico* approach and for *in vitro* findings as a future aspect.

MATERIALS AND METHODS

Sequence retrieval of gene targets

The nucleotide sequences of three targeted genes *PDCD-1*, *CTLA-4*, and *NY-ESO 1* were downloaded from NCBI (National Centre for Biotechnology Information) in FASTA format.

Design sgRNA of targeted genes

The sgRNAs of targeted genes were designed to use Benchling online software. In which downloaded nucleotide sequences were given where software designed the number of possible sgRNAs within its on-target activity (specificity score) and off-target activity (efficiency score) through CRISPR design and analysed guide tool in it.

Design immunogenic living drug

The lentivirus-HIV based CRISPR two vectors were selected from the Addgene home page to perform *in silico* cloning. Perform gene knock-out lentiCRISPRv2 and for insertion pCas-Guide-EF1a-CD4 selected. Along with it, designed sgRNAs were prepared as a gene of insert in cloning using SnapGene offline software. The restriction sites were created for both ends of sgRNAs according to their respective vectors. For *PDCD-1* and *CTLA-4*, to create sticky ends nucleotides 'CGTCTCACACC' (bases for BsmBI restriction site) on 5'prime and another end nucleotide, 'GTTTTGAGACG' (bases to create BsmBI restriction site on 3'prime) was inserted. Whereas for *NYESO-1*, BamHI restriction site on start 5' prime by adding nucleotide 'GGATC' to create BsmBI Restriction site on another end nucleotide 'TTTTGGAGACG' inserted. Then, by using restriction and insertional cloning tool of SnapGene *in silico* cloning of prepared *PDCD-1* and *CTLA-4* inserts with lentiCRISPRv2 vector and *NYESO-1* insert with pCas-Guide-EF1a-CD4 was performed.

In-vitro isolation of the lentivirus (pseudo-HIV within designed vectors, can be called immunogenic living drug) can be done by using a favourable host for example using HEK 293 (Human Embryonic Kidney 293 cells) derived suspensions cells. The Gibco LV-MAX lentiviral production system of Thermo Fisher SCIENTIFIC is the first optimized system that provides all the high quality components you need in a chemically defined, serum-free environment along with the protocol.

Analysis of protein-protein interactions

To analyse designed immunogenic living drug binding efficiency, protein-protein docking was performed using PatchDock online software. For that, HIV gp120 core protein, T cell receptor $CD4^+$ protein, and T cell receptor $CD8^+$ protein was downloaded from PDB (Protein Data Bank). Receptor (HIV

gp120 protein) was prepared by removing extra ligands and water molecules in BIOVIA discovery studio (Figure 1). The binding energy score as a binding efficiency was performed using PatchDock followed by FireDock.

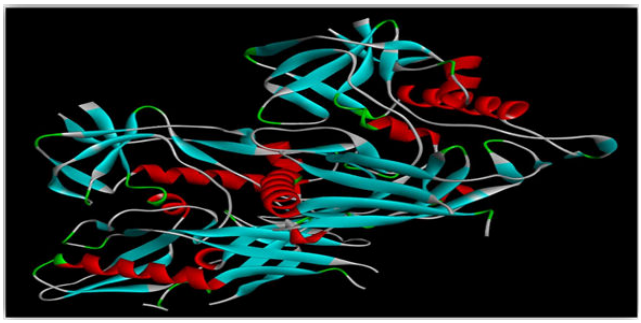

Figure 1: Modified structure of HIV enveloped core protein gp120.

RESULTS AND DISCUSSION

CRISPR/Cas9 genome editing tool is recognized as a powerful genome editing tool, enabling researchers to precise modification of genomic sequences, and helping change in target gene function in cure of many diseases. Cancer is now one of the popularly diagnosed diseases which can be cured by using the immunogenic living drug. Here, to design precise and impactful sgRNA for immunogenic cancer drug design, the following results are obtained.

Data retrieval based on the experimental requirement

The three genes were selected as the experimental targets. To target the genes for knock-out/knock-in using the CRISPR/Cas9 system the sgRNAs for them should be designed first. The FASTA sequences of all three genes (*PDCD-1*, *CTLA-4*, and *NYESO-1*) were downloaded from the NCBI gene databank to design sgRNAs.

Selection of designed sgRNAs

The sgRNAs for all three genes were designed by using the Benchling bioinformatics tool. The Benchling supplies all the possible sgRNAs for the selected target region on the genes. The starting regions sgRNAs were mostly selected for better specificity and efficiency because targeting end regions of the genes does not give proper efficiency and specificity for gene knock out/in [11]. From, all the designed sgRNAs, sgRNAs with specificity and efficiency scores nearby 100 were selected (Figure 2). In which the sgRNA for *PDCD-1* gene "CTATTTTGGAATCCACCCCG" was selected with its good specificity and efficiency score (Table 1). Likewise, the sgRNAs for *CTLA-4* and *NYESO-1* genes were "ACAGTGCTAAGGATGCTCAG" and "TTCTGACAGTTCTGGTGGCG" selected respectively (Tables 2 and 3).

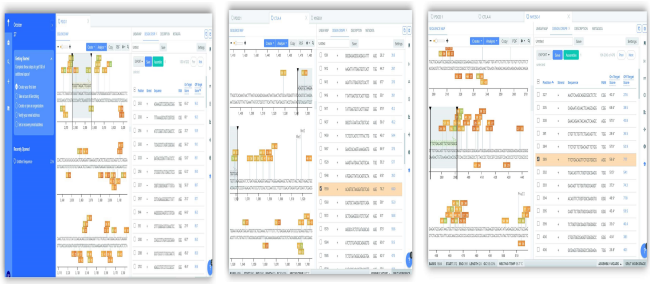

Figure 2: Selected *PDCD-1*, *CTLA-4* and *NYESO-1* sgRNAs with on-target and off-target scores near 100.

Table 1: Designed sgRNAs for *PDCD-1* gene, selected sgRNA highlighted in light blue.

Position	Strand	Sequence	PAM	Specificity score	Efficiency score
3760	-1	CTATTTTGGAATCCACCCCG	GGG	84.39	73.86
2553	-1	CTGTTTAAAAGCCACTCGGT	CGG	82.61	67.05
2161	-1	GAGAATTGCTTGAGCCCCGG	AGG	80.43	67.25
2994	1	TGGGGTTAGGACTTCCACAT	AGG	69.72	72.64

Table 2: Designed sgRNAs for *CTLA-4* gene selected sgRNA highlighted in light blue.

Position	Strand	Sequence	PAM	Specificity score	Efficiency score
1559	1	ACAGTGCTAAGGATGCTCAG	AGG	64.04	76.23

1693	-1	CTTCCACCAAGCCACTTGCA	CGG	63.62	60.23
1777	-1	CCAATTCCAATCTATCACTC	TGG	77.66	56.55
1798	1	AGATTGGAATTGGATCATGG	GGG	60.7	71.89
1927	1	ACTTGTCAGACTGACTGGAG	AGG	69.84	63.28
1949	1	GGGCCTGGTTAGTTACAGGA	AGG	67.22	66.65

Table 3: Designed sgRNAs for *NYESO-1* gene, selected sgRNA highlighted in light blue.

Position	Strand	Sequence	PAM	Specificity score	Efficiency score
389	1	TTCTGACAGTTCTGGTGGCG	AGG	77.65	56.61
461	1	GGAGATGCGAGTA AGTGGT	GGG	73.78	52.34
514	1	GATATGAGAGGCCAGCTGC	AGG	68	67.29

Lentivirus based immunogenic living drug designing

LentiCRISPRv2 is the lentivirus based vector consisting of CRISPR/Cas9 system into it along with pseudo-HIV regions which allow the formation of HIV viral coat, selected for knock out of *PDCD-1* and *CTLA-4* genes [12]. The pCas-Guide-EF1a-CD4 is also a lentivirus based vector consisting of CRISPR/Cas9 system, pseudo-HIV regions along with truncated *CD4* gene which allows knocking in by specific Cas9 enzyme. On besides of that, all three target genes were prepared by editing restriction sites on both edges of genes through SnapGene (Figure 3). And then restriction and insertional cloning were performed by using BsmBI restriction enzyme sites of LentiCRISPRv2 vector as well as by BamHI and BsmBI restriction sites of the pCas-Guide-EF1a-CD4 vector. The *PDCD-1* and *CTLA-4* sgRNAs were inserted into LentiCRISPRv2 and *NYESO-1* sgRNA was inserted into the pCas-Guide-EF1a-CD4 vector (Figures 4 and 5).

Figure 3: Edited *PDCD-1*, *CTLA-4* and *NYESO-1* sgRNA for insertion.

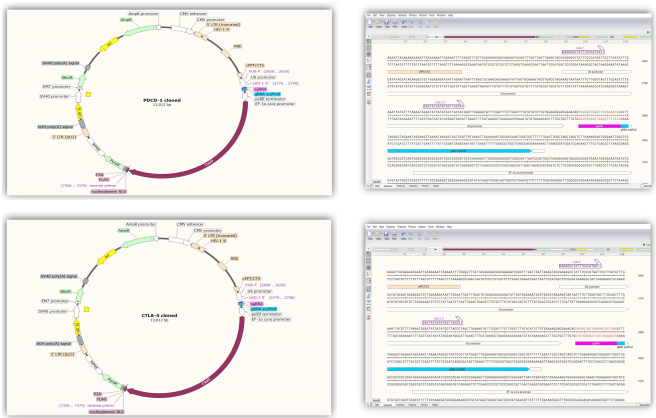

Figure 4: Cloned *PDCD-1* and *CTLA-4* gene into lentivrirusv2 cloning vector along with its sequence, sgRNA highlighted in a pink colour.

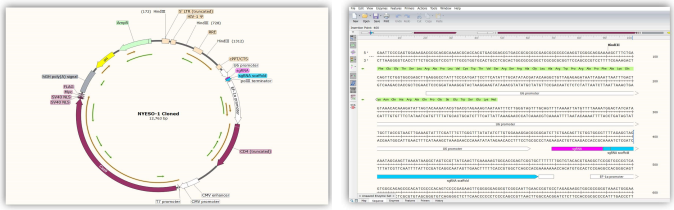

Figure 5: Cloned *NYESO-1* gene into pCas-Guide-EF1a-CD4 cloning vector along with its sequence, sgRNA highlighted in a pink colour.

Protein-protein docking

The protein-protein docking was performed to analyse protein-protein interactions along with their binding efficiency. The docking was performed by using PatchDock followed by FireDock online docking tools. PatchDock stands for the results according to three criteria:

- Molecular shape representation.
- Surface patches matching.
- Filtering and scoring and ranking the structures according to a geometric shape complementarity score [13].

The highest score stands for best complementarity of two proteins. The highest-ranked structure was selected for HIV gp120 and $CD4^+$ as well as HIVgp120 and $CD8^+$ on PatchDock. After that, among the results of PatchDock best 10 models were continued for FireDock. FireDock significantly improves the ranking of the rigid-body PatchDock algorithm by giving a binding score based on binding free energy (global energy), Atomic Contact Energy (ACE), softened van der Waals interactions, partial electrostatics, and hydrogen bonding [14].

FireDock automatically ranks the best Docking model for the inputs based on Global energy. The more negative value of global energy stands for a more specific binding of two proteins [15]. The HIVgp120 and $CD4^+$ shown -7.58 global energy value and HIVgp120 and $CD8^+$ shown -17.63 global energy value which stands for both $CD4^+$ and $CD8^+$ have good binding efficiency with HIV gp120 protein means both T helper cell and cytotoxic T cell receptors can interact with HIV envelop protein gp120 (Tables 4 and 5). The binding structure of both docked proteins opened in BIOVIA discovery studio was shown in Figures 6 and 7.

Table 4: Protein-protein docking result of gp120 and $CD4^+$ on FireDock.

Rank	Solution number	Global energy	Attractive VdW	Repulsive VdW	ACE	HB
1	3	-7.58	-38.18	34.75	14.95	-6.69
2	6	-3.75	-7.78	2.86	-3.79	0
3	4	28.28	-33.86	95.34	-2.32	-3.01
4	8	193.51	-56.83	331.43	14.05	-5.33
5	10	590.6	-41.73	811.59	-6.27	-1.59
6	9	1008.87	-51.71	1304.4	25.08	-8.94
7	1	1044.32	-70.88	1385.3	26.17	-8.86
8	5	2097.43	-66.85	2719.39	17.14	-6.04
9	7	2375.66	-55.93	3046.01	2.66	-9.15
10	2	3667.12	-93.99	4707.94	18.63	-10.81

Table 5: Protein-protein docking result of gp120 and $CD8^+$ on FireDock.

Rank	Solution number	Global energy	Attractive VdW	Repulsive VdW	ACE	HB
1	8	-17.63	-19.87	7.08	7	-2.88
2	1	-11.48	-23.79	16.39	3.96	-1.8
3	2	-4.99	-34.61	25.11	11.2	-5.1
4	6	0.6	-6.31	2.57	0.25	-0.82
5	7	3.93	-5.59	0.1	2.51	-0.48
6	9	6.99	-4.88	7.2	3.38	-1.26
7	10	20.89	-1.54	0	2.15	0
8	4	27.51	-19.47	37.78	14	-2.6

9	5	32.74	-23.26	8.33	16.17	-2.41
10	3	314.88	-40.68	470.26	-0.66	-7.08

Figure 6: Structure of docked proteins (i) HIV gp120 protein into blue, red, and green colour and (ii) T cell receptor $CD4^+$ into yellow colour.

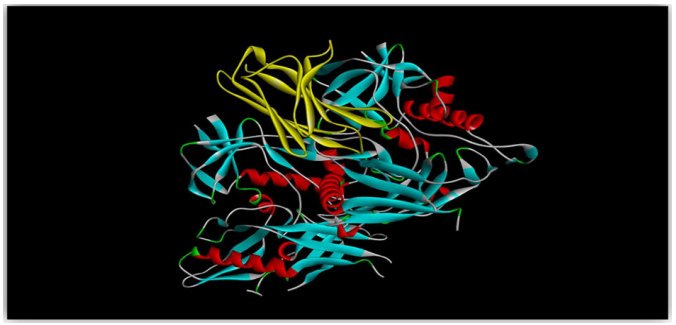

Figure 7: Structure of docked proteins (i) HIV gp120 protein into blue, red, and green colour and (ii) T cell receptor $CD8^+$ into yellow colour.

CONCLUSION

This research aimed to design sgRNAs to target specific genes *PDCD-1*, *CTLA-4*, and *NYESO-1* to activate helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) of the immune system against cancer. Based on in-silico analysis and findings, it can be concluded that the selection of the CRISPR/Cas9 delivery system is a key factor to consider when targeting tumour cells. The three sgRNAs were selected based on their specificity score: 84.39001, 64.0453326, and 77.657999 as well as efficiency scores: 73.863324, 76.237004, and 56.615160, respectively. The selected lentivirus based lentiCRISPRv2 and pCas-Guide-EF1a-CD4 vectors were shown successful *in-silico* cloning of designed three sgRNAs into the above vectors. They can be isolated easily *in-vitro* as an immunogenic living drug consisting of delivered clones as a genome of virus surrounded by HIV viral coat called pseudo-HIV. By analyzing protein-protein interactions of HIV coat protein gp120 and T cell receptors ($CD4^+$ and $CD8^+$), the study showed good binding efficiency as a global energy value of -7.58 and -17.63 respectively of the designed drug to the targeted immune genes and tumour antigen. The pipeline of this study will be the foundation for the *in-vitro* treatment of cancer.

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CONFLICT OF INTEREST

The authors proclaim no such conflict of interest.

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