

Different Variations and Applications of CRISPR-Cas System in the Field of Genetic Modification

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Abstract: CRISPR-Cas system originated from bacteria immune system and was discovered in *E. coli* in 1987, but the turning point of CRISPR technology was in 2020 when Doctor Emmanuelle Charpentier and Doctor Jennifer Doudna discovered how to manipulate the crRNA and tracrRNA in CRISPR forming crRNA. CRISPR-Cas system can be used in different fields, such as biomedicine and genetic modification. In this research, three types of CRISPR-Cas system in gene editing and immune therapy is discussed. HAVE-a CRISPR-Cas3 based detection tool can be used to lethal variant of HIV, HIV16 and HIV18, in hopes to detect HIV early to more effectively treat patient and prevent HIV induced cancer. Being the first approved and available to the market CRISPR-Cas based medicine is a major breakthrough. Cas10 COVID-19 detection was developed during the COVID-19 pandemic time period for a fast cheap and mass test for COVID-19. As a substitution for qRT-PCR, Cas10 has the advantage of being a faster and more specific alternative to qRT-PCR.

Keywords: CRISPR-Cas, genetic modification, immune therapy, disease treatment

1. Introduction

Genetic modification is widely used nowadays as an effective and relatively safe methods to treat genetic disorders, with a significant breakthrough in recent history being the discover of the CRISPR-Cas system. The common structure of a CRISPR-Cas system consists of a leader sequence followed by an array of short repeated sequence that interspaced by viral genetic material known as spacers, also a set of Cas. Preceding the Cas protein is a tracrRNA, which encodes a non-coding RNA that is complementary to the viral DNA acting like an anker for the Cas to use its domain to cleave the viral DNA into shorter fragments [1, 2]. The fragments then are incorporated in the form of “spacers” forming a CRISPR array, a long strand of DNA that has viral DNA and the bacteria’s own DNA. Once the new spacer is incorporated allowing transcription to take place forming a strand RNA called pre-crRNA complementary to the CRISPR array, the pre-crRNA then continue binding with the tracrRNA forming a complex. The RNA complex is cut by RNase3 to produce mature crRNA. When the same viral DNA invades the host, the mature tracrRNA-crRNA complex binds with the Cas9 protein forming RNP. RNP is used as a guide for Cas9 protein to the protospacer adjacent motif allowing the crRNA to recognize and bind with the viral gene performing a site-specific double strand cleavage, destroying the viral DNA sequence.

The increase in popularity of CRISPR-Cas system is largely because of its simplicity and incomparable accuracy, and the common structure of a CRISPR-Cas system only relies on a two-part system as mentioned before a Cas protein and a complex of sgRNA [3, 4]. The CRISPR-Cas system

can be classified into 2 class with each class having 3 types. Class 1 system relies on multiple different Cas protein, and in contrast Class 2 only requires one Cas protein. The most well know and utilized is CRISPR being CRISPR-Cas-9 given their unique and practical properties in genome and transcriptome manipulation [1]. They are widely researched and CRISPR and its associated protein subunit “Cas-9” has been extracted from the host, coming from *Streptococcus pyogenes*.

CRISPR-Cas system follows the general framework of its predecessor gene-editing tools. In comparison CRISPR-Cas system, it uses synthetic sgRNA and is not protein domain to induce the required Homology to target double strand breaks (DSBs) [5]. In addition to the difference in gene editing mechanism, CRISPR-Cas system is shown to be highly modifiable not only in the design of sgRNA also in the synthesis of Cas protein, fine tuning each Cas protein to perform unique functions. Due to CRISPR-Cas system’s moldability, the system is the focus in research for developing effective treatment for cancer, HIV, and array of disease, and fragile X syndrome disorders through immune therapy and gene manipulation [1]. Therefore, this research will discuss the different variations and applications of CRISPR-Cas system in the field of genetic modification.

2. Applications of CRISPR-Cas system

Although the CRISPR-Cas system has only been discovered for 13 years, there are already 9 different CRISPR-Cas based drugs/treatments under development by 2024, with the first CRISPR-based drug already approved in 2023.

Chronic bacterial infection is also becoming a target for the CRISPR-Cas system, and urinary tract infections (UTIs) are a common infection that affects woman causing more than 8 million visits to health care providers annually. Locus Bioscience has completed the development of a treatment targeted towards chronic bacterial infection and currently are in clinical trials, and the treatment is a combination of 3 different bacteriophages engineered to incorporate CRISPR-Cas3 within themselves. Diseases induced by fault protein folding are caused by mutations in the base sequence of DNA, resulting in the synthesis of fault proteins. The treatment currently in research involves the usage of Cas9 to reduce the count of faulty gene within the patient’s DNA. In total, there are 2 class and 6 type I-VI of and several subtypes of CRISPR-Cas systems. However, the key systems that commonly used in gene-editing and immune therapy are Type II (Cas9), Type IV (Cpf1/Cas12) and Type (C2c2).

2.1. Type I cas3

Type 1 CRISPR-Cas3 being part of Class 1 CRISPR=Cas’s, is an RNA-guided multi-Cas protein complex [2]. Compared to type 2 CRISPR-Cas9, it does one pair of precise cuts on the targeted gene. CRISPR-Cas3 detects and degrades exogenous or targeted genes through the helicase nuclease-Cas3 protein, and the rapid degradation process is called Cascade.

The looped collateral single-strand DNA (ssDNA) nuclease activity within class 1 type I CRISPR Cas system has been used [3]. Because the hyperactive ssDNase activity of Cas3, this can lead to many false-negative diagnoses. Intern the team have identified and used a Type I-A Cas3 variant TsiCas3 collected from *Thermococcus sirci*, TsiCas3 remained dormant due to its auto-inhibition mechanism [3]. Structural analysis showed the electrostatic and the conformational shift, and a critical glutamate residue (E217) in TsiCas3, was able to suppress ssDNA binding, and only until the formation of R-loop the function of the Cas3 is reactivated. In addition, TsiCas3 exhibited the ability to recognise broad PAM sequence on the viral genome. Recognising a 5'-Y-Y-N motif (Y = Cytosine/Thymine) allowing TsiCas3 to target 16 PAM sequence in comparison to other systems it is much broader [3].

For the ability to tolerate mismatched sequence of TsiCas3, mismatch in this context indicates failure of complementary base pairing that effected the system functionality. To evaluate mismatch tolerance, two distinct target DNA sequences sharing the same 5'-CCC PAM motif was used. For each target, in total 110 variants were tested 55 mismatched variant, 37 single mismatches and 18 clustered mismatches (double/triple mismatched). The Collateral ssDNA cleavage activity was monitored through fluorescence markers. Mismatches in PAM-proximal seed region (the first 24 bases) showed significant reduction in activity. Out of the 110 variants tested the 37 single mismatches causes significant dysfunction, in contrast the 18 clustered mismatches nearly abolished the activity of the first 16 base [3].

Tolerance every 6 base, mismatch at every 6 bases for all 110 variants showed no effect. The reason for no effect is due to Cascade complex particularly the Cas7 protein, and the crRNA is bounded by a helical arrangement of Cas7 subunit. At every 6th position the subunit physically sequestered the crRNA, it can prevent the RNA from binding with the targeted DNA [3]. Mismatches over 24 bases from PAM showed well toleration have minimal effect to cleavage efficiency (Figure 1).

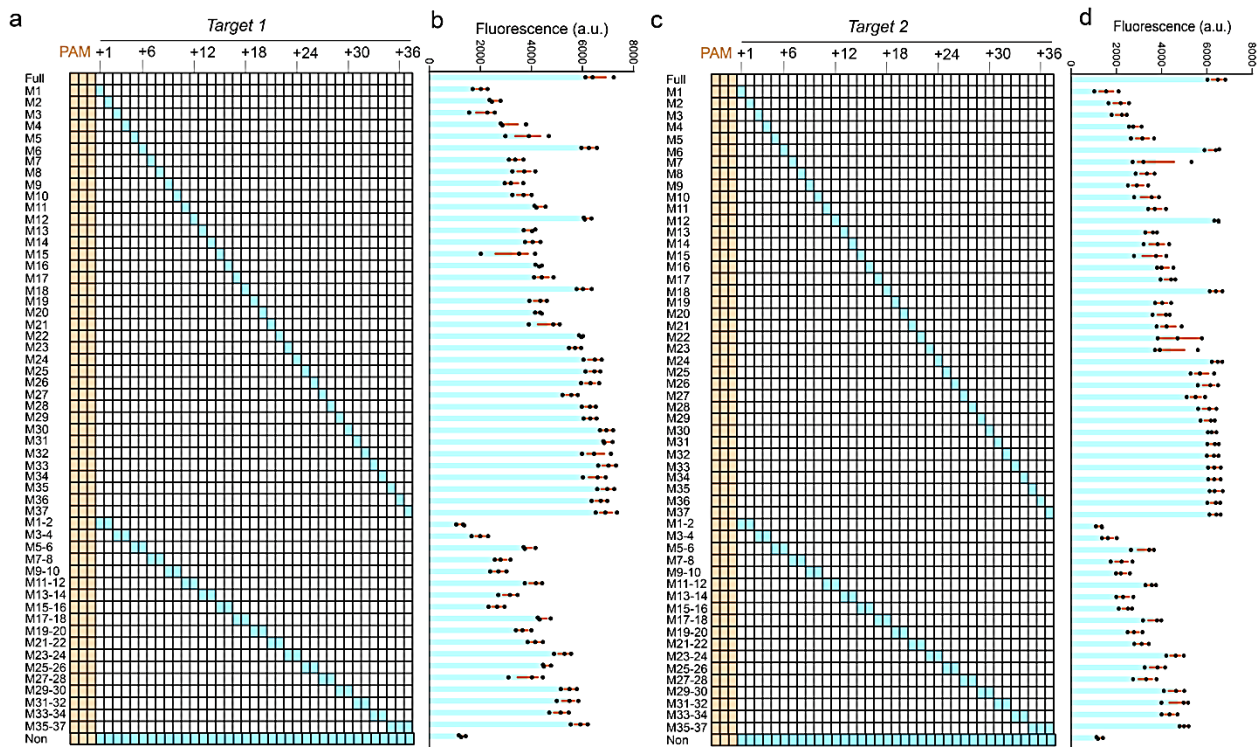


Figure 1: Mismatches over 24 bases from PAM [3]

TisCas3 also showed dual activation mode, having the ability to respond to both DNA and RNA targets, increasing the diagnose range (e.g. RNA viruses like SARS-CoV-2). This ultimately leads to the development of Hyper-Active-Verification-Establishment (HAVE) [3], where TsiCas3 can be used for precise detection of high-risk subtypes of Human Papillomavirus (HPV) HPV16/HPV18, a leading cause for cervical cancers. HAVE integrates isothermal RPA amplification that is a technique for amplification of specific DNA sequence at a constant temperature, which unlike traditional techniques that require consistent heating and cooling is much straighter forward. In the case of HAVE, only 10 min is required to generate the target amount of DNA for testing of HPV [3]. And the other component of HAVE would be TsiCascade-Cas detection, and the specifically engineered Cas3 complex cleaves a fluorescent marker, and generating a signal within 15 min [3]. The test was

carried out on 30 clinical samples HAVE showed 100% accuracy in distinguishing between the two HPV variant [3]. Compared to “gold-standard” PCR-RDB, it only showed 93.3% accuracy for HPV16 and HPV-18 with only one false positive result. HAVE was able to distinguish between different HPV subtypes, showing HAVEs high specificity and accuracy.

Hyperactive ssDNase activity of Cas3 was overcome by substituting it for a variant of TisCas3, which would only be activated by presence of R-loop. TisCas3 showed low mismatching in PAM region, every 6 bases away from PAM and every 24 bases, with dual activation responding to both DNA and RNA, leading to HAVE demonstrating successful in generating necessary volume of DNA for detection of HPV18 and HPV16 variant in a short period of time.

2.2. Type II Cas 9

CRISPR-Cas9 is undoubtedly the most important discovery and innovation and discovery in the field of genetic engineering in 21st century. The discovery of CRISPR-Cas dates to the 1970s where Japanese scientist discovered the first CRISPR-Cas in bacteria [6, 7]. The power of CRISPR-Cas system-based gene editing therapy is its flexibility and moldability for specific patient that is customized to their case [6]. Recently, Casgevy has passed clinical trial phase 3, being the first ever Cas9 genetic editing-based treatment medicine that is approved this is a breakthrough in the field of gene therapy. Casgevy is a medicine use for treating transfusion-dependent beta-thalassemia (TDT) and sickle cell disease (SCD). Both TDT and SCD are the most common monogenetic diseases worldwide, the combination accumulated for approximal of 360,000 patients annually [4]. Sickle haemoglobin is caused by a point mutation. Changing the codon from GAG producing glutamic acid to GTG produces valine [5], and the substitution of the amino acid valine for glutamic acid occurs at the 6th position of the beta-globin chain. Changing the shape of hemoglobin, hemoglobin sticks together to form sickle shaped red blood cells in a low oxygen environment.

Polymerization of deoxygenated sickle shaped red blood cell causes patients symptoms such as anemia, periodic episodes of extreme pain caused from vaso-occlusive crises (blockage of artery's due to the blood cells sticking together preventing tissue from obtaining oxygen from blood), hemolysis, organ damage, and usually reduced life span [5]. Prior to the development of Casgevy, the options for treating SCD and TDT were limited, and hematopoietic stem cell transplantation (HSCT) was an effective method among many failed treatments. However, HSCT still came with many limitations such as restricted by donor compatibility and long-termed autoimmune side effects.

CD34+HSPCs was collected from two patients a 19-year-old female with TDT expressing β^0 and β^+ genotype and a 33-year-old female with SCD expressing β^S and β^S genotype. CRISPR-Cas9 was used to disrupt the targeted BCL11A, where BCL11A is a transcriptional factor repressing γ -globin expression and fetal haemoglobin (HbF) expression [5]. In autologous hematopoietic stem and progenitor cells (HSPCs) [5], it is aiming to reactivate synthesis of Hbf. Preclinical trial data shows 80% allelic editing efficiency in healthy donor cells [5], with no noticeable off-target effect. Patients undergo myeloablative condition (Busulfan) and then received infusions of the CRISPR Cas9 edited CD34+CTX001 [5]. TDT patient achieved transfusion independence within 30 days after receiving CTX001 HbF levels rose from 0.3 g/dL (baseline) to 13.1 g/dL at 18 months, where is about 99.7% of all erythrocytes expressing Hbf [5].

SCD patients did not experience vaso-occlusive episodes during 16.6 months of treatment with CTX001. HbF concentration was from 9.1% to 43.2% and a near-complete pancellular erythrocytes HbF expression. Both patients showed durable engraftment in their bone marrow towards edited HSPCs, exhibiting high editing rates >60% maintained in bone marrow and blood stream even after 1 year [5]. Although there was great success in the clinical testing of Casgevy, there was still some adverse effects, such as veno-occlusive liver diseases, pneumonia with Neutropenia, and iron overload but all the adverse effect was quickly managed and solved. Pancellular HbF distribution was

near complete >99.9% [5], confirming edited HSPCs increases the synthesis and lineage of erythroid drastically, therefore reducing sickle red blood cell in SCD, improving erythropoiesis in TDT and giving patients a higher chance of survival. The stable allelic editing in bone marrow at 12 months shows long term engraftment. In addition, there was no off target detected.

2.3. Type III CRISPR Cas III

Type III CRISPR Cas III is multiprotein effector RNA to detect CRISPR-Cas complex. The complex is known as the Cas-10-Csm complex, being a multiprotein effector, and it is composed of on crRNA and 5 Cas protein: Cas10, Csm2, Csm3, Csm4 and Csm5 [8]. When foreign RNA transcript is detected, type III is activated causing a secondary messenger cyclic oligoadenylates (cOAs) produced from Palm-2 domain (converting ATP into cOAs).

Type III-A (Csm complex/III-A) is a subtype of Type III CRISPR-Cas. It is a DNA targeting via cas10 protein complex triggered by RNA binding and can trigger downstream immune responses through production of signaling molecules cyclic oligoadenylates. Different Cas protein can result in a different function being produced by the CRISPR complex for instance Csm6 a protein not part of Cas10-Csm complex. When bound to type III, it deactivates the cell into dormancy, thus blocking the viral replication [9].

Type III-B (Cmr complex/III-B) is RNA targeting Cas complex. Like type-A, it also utilizes cas10. With the addition of Cmr proteins, type B directly cleaves into pathogen RNA and can trigger collateral RNase, causing downstream immune response. During COVID-19 pandemic, there was an urgent need for the development of a fast, cost-effective diagnostic tool. Although the golden stander of qRT-PCR remains, its limitation such as slow result and specialised equipment has led to the research of CRISPR-based detection method. Type III CRISPR-Cas10 has been noted with the potential of accurate RNA detection by Cas10 because of synthesis of cOAs [10]. Type III CRISPR complex was combined with SARS-CoV-2 RNA along with cOA synthesis. Cas10 polymerase was activated when crRNA recognized the viral RNA [10]. cOA was synthesized and was able to stimulate the RNase activity of Csm6 or a Csm6 homolog, leading to the Csm6 cleaving a fluorophore-quencher reporter RNA. The 10 crRNA was synthesized, targeting the conserved area of SARS-CoV-2. A conserved area is referred in protein or genome as the sequence or area that is relatively unchanged through evolution. During testing, the crRNA was cross tested with other coronaviruses such as SARS-CoV-1, and the results showed no false positive recognition.

Multiplexing is referred as multiple crRNA, targeting different area of genome. After multiplexing, the sensitivity of crRNA guides recognising direct RNA increased from 10^7 to 10^6 copies/ μ L [10]. Yet for Cas10 used as a viable detection tool, its sensitivity was still not enough. The integration RT-LAMP amplification+T7 transcription (T7 Csm) increased the LoD (limit of detection) of 200 copies/ μ L within 30 minutes, making it a viable detection tool [10].

With nasopharyngeal swabs, the results from CRSPIR-Cas10 complex showed 100% specificity with no cross reactivity between SARS-CoV-1, MERS-CoV or other pathogens [10] and 74% sensitivity for samples with viral loads ≥ 200 copies/ μ L. In addition, The Cas10 complex was able to detect B.1.1.7 (Alpha) variant of SARS-CoV-2 [10], further showing its adaptability. Comparing DETECTR and SHERLOCK both FDA approved CRISPR diagnostics tool, Cas10 offered faster result 30 VS. 45-50 minutes at a slightly lower sensitivity [10].

CRISPR-Cas10 was selected as a new detection tool for viruses such as COVID-19 due to its RNA detection nature via synthesis of cOA. 10 crRNA designed to target COVID-19's conserved area for accurate result and multiplexing alongside of RT-LAMP+T7 Csm was used for increases sensitivity. The results showed high sensitivity with no false positive between different coronaviruses. Although Cas10 had slightly lower accuracy, it gave faster result compared to FDA approved CRISPR diagnostics tool.

3. Conclusion

HAVE can be used to achieve precise lethal HIV variant HIV16 and HIV18 detection using Cas3, cover coming the difficulty of hyperactivity of Cas3 by opting for a variant of Cas3. As mentioned previously, TisCas3 was only activated in the presence of R-loop. And during testing, it showed excellent tolerance for mismatch. In addition, compared to “gold-standard” PCR-RDB, HAVE showed 100% accuracy in distinguishing between the two HPV variant. Casgevy-first CRISPR-Cas based medicine can be used to treat TDT and SCD. Cas modified HSPCs were given to patient, and the modified HSPCs had its fetal haemoglobin repressing gene removed, therefore fetal haemoglobin was produced replacing the mutated sickle shaped red blood cell. Cas10 COVID-19 detection was developed during the COVID-19 pandemic period. As a substitution for qRT-PCR, Cas10 synthesizes cOA and are designed to target conserved area of COVID-19 alongside RT-LMP+T7 CSM, where Cas10 demonstrated a faster and more specific alternative to qRT-PCR. With the help of CRISPR-Cas system, human now is given the opportunity to edit the fundamental building block of living organism, being DNA therefore allowing illnesses to be cured through the precise and effective regulation of disease-causing genes or the permanent correction of harmful base mutations. CRISPR-Cas has the power to edit our own DNA overcoming the limitation that was bounded by technology in the past. Overall, the three studies’ findings demonstrated that CRISPR-Cas has enormous promise in a wide range of application and the 3 analysed in this research is only a fraction of its true potential.

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