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The development of CRISPR/Cas Technology in Potential Clinical Applications

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The development of CRISPR/Cas technology ~~in~~for potential clinical applications

Yueying Huang *et al*. Application of CRISPR Technology

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Abstract

Clustered regularly interspaced short palindromic repeats~~, and their effector proteins~~ (CRISPR~~)~~), their effector proteins, and CRISPR-associated (Cas) (CRISPR/Cas) proteins constitute ~~an~~the innate adaptive immune system ~~found~~ in several bacteria and archaea ~~owing to long-term evolutionary changes and can resist~~. This immune system helps them in resisting the invasion of phages and foreign DNA by providing sequence-specific acquired immunity. ~~Due~~Owing to the numerous advantages like ease of use, low cost, high efficiency, good accuracy, and a diverse range of applications, the CRISPR-Cas system ~~encompassing tools like gene-editing and molecular diagnosis~~ has become the most widely used genome editing technology ~~in molecular biology~~. Hence, the advent of the CRISPR/Cas technology highlights a tremendous potential in clinical diagnosis and can become a powerful asset for modern medicine. This paper reviews the recently reported application platforms for screening, diagnosis, and treatment of different ~~clinical~~ diseases based on CRISPR/Cas systems ~~while summarizing the~~. The limitations ~~and~~, current challenges, and ~~looking forward to its application prospects; thus, providing~~future prospectus are summarized; this article would be ~~–~~a valuable reference ~~as well as a therapeutic strategy~~ for ~~opening new avenues in~~ future genome-editing practices.

Keywords: CRISPR-Cas; Gene editing; Molecular diagnostics

Core tip:

~~Based on the rapid development of CRISPR/Cas technology, this~~This review mainly discusses and explores ~~its~~the potential clinical applications. ~~Detection~~ of CRISPR/Cas

technology. The detection technologies offor nucleic acids and small molecules of different pathogens based on the CRISPR/Cas system are summarized while. The advantages and disadvantages of the CRISPR/Cas technology from the aspects of gene editing, disease treatment, multi-drug resistance analysis, and treatment are enumerated, which makes it convincibleasserting that CRISPR/Cas system has unlimited potential andin clinical applications with certain challenges in clinical application.

INTRODUCTION

The CRISPR/Cas system, an adaptive immune system present in numerous bacteria and archaea, is a nucleic acid-targeted defense mechanism composed of numerous short and conserved repeat regions and spacers that protect ~~itself~~themselves from exogenous mobile genetic elements such as plasmids and phages~~; a~~. A similar arrangement in the chromosomes of Gram-negative bacteria (*E. coli*) was also reported by Ishino Y et al. in 1987 and was again confirmed in ~~a~~ 2010 ~~study, that the~~. The type II CRISPR/Cas system from *S. pyogenes* could specifically recognize and cleave target DNA guided by gRNA[1]; thus, laying the foundation for the development and utilization of the CRISPR/Cas system ~~by posterity.~~. Over the past few years, several CRISPR/Cas systems belonging to Cas proteins with different characteristics have been developed, which in turn have produced many CRISPR/Cas system-related toolboxes ~~due to~~, offering functional robustness, efficiency, and ease of implementation in multiple organisms[2]. Various gene-editing tools ~~represented by~~based on CRISPR/Cas9 were ~~first~~ introduced in 2013[3], followed by successful implementation in the modern medical field ~~by virtue of its convenient operability and efficient editing ability.~~. Within a few years, CRISPR/Cas technology ~~has~~ gradually made crucial breakthroughs and is now widely employed in ~~the fields of~~ gene-editing, treatment of genetic diseases, clinical diagnosis of common pathogenic molecules, ~~as well as measures for~~and alleviating antimicrobial resistance. This review ~~mentions~~compiles the recent ~~advent of~~advancements in CRISPR technology ~~in modern medicine while summarizing~~ and ~~discussing~~summarizes the achievements of CRISPR/Cas technology in clinical ~~application as well as the laboratory research results of potential clinical~~applications to provide ~~substantial~~ opportunities for programmable

genomic editing ~~so that it can become a valuable asset~~ for translational medicine.

CLASSIFICATION OF CRISPR/CAS SYSTEM

~~Along with the deepened study on~~In CRISPR/Cas systems, researchers have ~~disclosed~~discovered various Cas proteins with different characteristics. There are two classes of CRISPR/Cas systems ~~determined by~~based on the composition of their effector subunits, ~~Class~~Classes 1 and 2~~, in which~~. Class 1 system contains numerous RNA–effector complexes, ~~whereas class~~and Class 2 system comprises of a solitary protein like Cas 9 that conducts all effector complex activities[4]. Class 1 CRISPR–Cas system includes type I, III, and putative IV subtypes. The type I subtype contains the signature gene Cas3 encoding a single-stranded DNA (ssDNA~~)~~), which acts as a candidate for guiding cascade in type I CRISPR module like Cas8 for DNA invasion, but this theory has not been thoroughly investigated. Type III CRISPR-Cas system containing gene Cas10 includes nonspecific degradation of both ssDNA and RNA molecules ~~while requiring~~and requires the target DNA transcription for immunity[4, 5]. The Cas10 subunit cleaves the transcribed ssDNA and activates the Csm6 nonspecific-RNase activity, while as the Csm3 cleaves the RNA target, the RuvC domain introduces staggered dsDNA breaks[6-8]. Since the Cas10 subunit HD domain cleaves ssDNA from the transcription bubble[9-11], due to activation of the Palm domain, ATP is converted into four or six-member CoA rings~~, which~~. These six-member CoA rings then ~~acts~~act as a secondary messenger and ~~activates~~activate Csm6 by binding to its CARF (CRISPR-Associated Rossman Fold) domain, causing activation of the HEPN domain~~,~~ and unleashing nonspecific RNA cleavage[12-15]. Putative Type IV CRISPR/Cas systems include a large subunit, Csf1, Cas5, and Cas7 (a solitary unit), and usually lack the known Cas proteins involved in adaptation and target cleavage[16].

Class 2 effectors are composed of a single Cas unit that associates with the crRNA for gene targeting in various biotechnological applications. This class includes CRISPR types II, V, and VI. Type II uses the mature crRNA directed CRISPR-associated protein Cas9 base-paired to trans-activating crRNA (tracrRNA) while introducing double-stranded breaks (DSB) in target DNA. Although Cas9 also harbors RuvC and HNH domains, at sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas. In contrast, the Cas9 RuvC-like domain cleaves the noncomplementary strand, thereby stressing the fact that target recognition by Cas9 requires both a seed sequence in the crRNA as well as a GG dinucleotide-containing PAM sequence adjacent to the crRNA-binding region in the DNA target[1, 16-20]. The majority of type V CRISPR modules can recognize dsDNA targets. For instance, a single CRISPR RNA (crRNA) processed by Cas12 RNase domain guides Cas12 with a T-rich protospacer adjacent motif (PAM) sequence to cleave double-stranded DNA (dsDNA) targets, generating sticky ends[21]. Cas12a cleaves both the target and non-target strands of a targeted dsDNA by a single active site in the RuvC catalytic pocket[22]. Besides Cas12, Cas14 is the smallest RNA-guided nuclease discovered to date with 400–700 amino acids and does not require a target sequence such as protospacer adjacent motif or PAM in the ssDNA substrate[23]. Lastly, Cas13 (also named as C2c2) belongs to Typetype VI recognizing RNA targets. A study discovered that Cas13 has two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains commonly associated with ribonucleases (RNases)—one for cutting its RNA target and the other one for processing the CRISPR-RNA (crRNA)[24-26]. It is also suggested that both type V and type VI CRISPR modules work collaterally, i.e., Cas12 and Cas14 can cleave ssDNA nonspecifically, while.

In contrast, Cas13 can initiate nonspecific RNA cutting,[16] which has not been observed when Cas12, Cas14, and Cas13 have been applied in either human or plant cell lines[27, 28].

CLINICAL APPLICATION OF CRISPR/CAS TECHNOLOGY

Application of CRISPR/Cas technology in clinical disease diagnosis

Application of CRISPR/Cas technology in pathogen detection

A 2017 study by Zhang Feng et al. (2017) developed a detection platform called Specific High-sensitivity Enzymatic Reporter Unlocking (SHERLOCK) that combined isothermal amplification and Cas13a technology for the detection of either single DNA or RNA molecule. It was reported that asAs activated Cas13 cleavedcleaves quenchable fluorescent RNA, producingit produces a quantifiable signal indicating the presence of the target nucleic acid;. SHERLOCK was demonstrated to detect closely related Zika virus and dengue virus due to its property of rapidly detecting nucleic acids with high sensitivity[26]. Subsequently, SHERLOCK was updated to SHERLOCKv2, based on simple four-channel multiplexing with orthogonal nucleic acid sequences of LwaCas13a, PsmCas13b, CcaCas13b, and AsCas12a[29], breaking through the quantitative and fluorescence limitations of SHERLOCK. Due toSHERLOCKv2 uses fewer primers used during the pre-amplification process to achieve better quantification without affecting sensitivity. Further, and the introduction of the test strip to determineaids in determining whether the target DNA or RNA is present in the sample by visualization-. SHERLOCKv2, due to its efficiency, specificity, ease of use, and portability, can detect ssRNA of dengue or Zika virus, as well as mutations in liquid biopsy samples from patients by the lateral flow assay system. Doudna Jet al. also reported that Cas12a could be used as a potential

nucleic acid detection platform and developed DNA endonuclease-targeted CRISPR trans reporter (DETECTR),[30] whose working principle was similar to that of SHERLOCK as this platform used isothermal amplification and Cas12a ssDNase activation for detection ~~which~~. Their system could specifically identify two human papillomavirus strains (HPV16 and HPV18) from human SiHa and HeLa cells with higher accuracy.

However, as the ~~above~~ above-mentioned nucleic acid detection processes require repeated uncapping and extraction, Zhang Feng et al. introduced the "one-pot" detection technology ~~in 2020~~, while updating the COVID-19 detection technology, in which RNA does not need to be purified from patient samples~~, and~~. Moreover, the reaction steps required to detect COVID-19 are done in a tube during the test~~, which~~. It was then named SHERLOCK Testing in One Pot~~,~~ (STOP)[31]. While STOP employs loop-mediated isothermal amplification (LAMP) as a method ~~of amplifying~~to amplify RNA and utilizes AapCas12b, an enzyme that can remain active at 60 °C (the temperature required for the LAMP reaction), the steps of RNA extraction were simplified enough that could easily detect viral RNA only by adding viral lytic releasers to throat wipes or saliva samples containing new crown viruses without purifying and isolating RNA.

Although STOP simplifies the complexity of the detection process and reduces the contamination caused by reagent transfer, it also requires heating, amplification, and other steps when compared with SHERLOCKv2. For overcoming these hindrances, a new system, CRISPR-Cas9-assisted DNA detection (CADD), was developed ~~containing~~. This system employed the Beads-HCR method, in which a pair of dCas9-sgRNAs, after being attached to the target DNA, was captured by the bead surface, followed by the addition of two hybridization chain reaction (HCR) hairpins (hairpin 1 and hairpin 2). Since the

hairpin was labeled with fluorescein, ~~so~~ HCR could produce a fluorescent signal on the bead's surface. As the hairpin was connected continuously, ~~and~~ the fluorescence signal became brighter in the positive direction, thereby making it easier ~~for detecting~~ to detect the fluorescence signals to the greatest extent without amplifying the target gene[32].

Due to the advantages of simple design, high efficiency, convenience, and a wider scope of application, CRISPR-Cas systems have become the most frequent genome editing technology in molecular biology while simultaneously promoting the development of basic scientific research, molecular innovations, and advanced clinical approaches. Zhang Feng et al. combined the Cas13 antiviral activity with its diagnostic ability and established a powerful and rapidly programmable diagnostic and antiviral system, named Cas13-assisted restriction of viral expression and readout (CARVER~~), for detecting~~). This system could detect RNA-based viruses like influenza A virus in human cells within two hours ~~by checking detection results~~ on the test strip itself[33]. Doudna ~~J~~ et al. also developed a COVID-19 detection technology connected to a smartphone that took only 15–30 min from sampling to reporting results on a mobile phone ~~as this~~. This technique omitted the reverse transcription and pre-amplification steps and used CRISPR to directly detect viral RNA ~~as the~~. It was a new diagnostic test that, apart from producing positive or negative results, could also ~~measured~~measure the viral load in a given sample[34]. Since Csm6 can sense the presence of RNA small loops and cleaves various RNA molecules, Doudna et al. utilized Cas13, Csm6, and their activators in combination to create a tandem nuclease method for detecting COVID-19~~, avoiding~~. This method avoided the original amplification of RNA and the possibility of sample cross-contamination caused by amplification[35].

Application of CRISPR/Cas technology in tumor pathogenesis monitoring

Tumor ~~incidence~~ monitoring is still an important parameter of global chronic disease monitoring. In recent years, due to ~~the disadvantages of~~ complicated operations, long detection time, and low specificity, genetic sequencing ~~while conducting~~for tumor marker detection ~~is not progressing normally.~~has lost its popularity. For overcoming this recurrent issue, Chow et al. developed in vivo CRISPR screening ~~in vivo~~[36] that identified both oncogenes and tumor suppressors that are the regulators of tumor immunotherapy in the tissue microenvironment[37] ~~and~~. This method could be suitable for personalized cancer modeling and ~~tumor~~ tumor-driven analysis in the future to provide guidance for precision medicine. In recent years, as miRNAs have been reported to be associated with tumorigenesis, diagnosis~~as well as~~, and prognosis, the CRISPR/Cas technology promises great potential for an early diagnosis of miRNA-related disease[38, 39]. Qiu et al. developed the RCA-CRISPR-split-HRP ~~system~~ (RCH) detection system based on dCas9, which ~~has achieved the application of~~facilitated CRISPR/Cas9 technology in miRNA detection for the first time[40]~~, with~~. It offers great advantages like a low detection cost and significant genetic effects. For demonstrating the potential application value of RCH, it was applied to detect circulating let-7a in serum samples from patients with non-small cell lung cancer (NSCLC) and healthy volunteers ~~as~~. As it was reported that since the expression of circulating let-7a was significantly down-regulated in patients with non-small cell lung cancer, it could become a useful biomarker for NSCLC[41]. It was also discovered that the detection results were highly consistent with RT-PCR results ~~as well as the contents reported in~~and the literature reports, suggesting that this method could be used for the screening and diagnosis of tumors in the near future. Simultaneously, Lee et al. developed

CRISPR-mediated, Ultrasensitive Detection of Target DNA)-PCR (CUT) using Cas9/sgRNA for specific cleavage of wild-type DNA in blood samples from colorectal cancer patients[42], which in turn enriched the circulating tumor DNA content in plasma to improve the specificity and sensitivity of early tumor diagnosis. While the SHERLOCK and SHERLOCKv2 molecular detection platforms can detect the BRAF V600E mutation in simulated circulating DNA samples and the EGFR L858R mutation in liquid biopsy samples from adenocarcinoma patients[26, 29, 43], the DETECTR method can also be used to rapidly detect tumor mutations in the reproductive system[30], for. For example, human papillomavirus, closely related to the occurrence of cervical cancer, is detected in human anal wipe DNA extracts[41], while the. The development of this technology is expected to bring a significant breakthrough in the early screening of cervical cancer.

Application of CRISPR/Cas technology in gene-editing disease therapy

In clinical medicine, gene-editing has changed from a niche research technique to an extensive and highly precise tool for disease treatment,[44] which has shown surprising results in the treatment of a variety of clinical diseases, such as genetic disorders[45] and cancer immunotherapy[46].

Application of CRISPR/Cas9 System in gene editing disease therapy

An in vitro study by Feng Z et al. observed that Cas9 protein could target and edit genes in bacterial cells, human stem cells, zebrafish, and human cell lines[47]. In the targeting phase, as Cas9 is guided to the DNA target site beside the PAM (spacer sequence adjacent motif) site (3 'GGN) by gRNA, the. The Cas9 nuclease domains like RuvC and HNH cleave single strands of DNA to form DNA double-strand breaks (DSBs), respectively.).

Although non-homologous end joining (NHEJ) is subsequently activated in the host for repair, ~~forming~~resulting in frameshift mutations, the body can also perform precise repair by homologous recombination repair (HDR) in the presence of homologous sequences[48]. Lately, the CRISPR/Cas9-mediated gene therapy has become a quick and effective gene-editing tool as it corrects single-gene mutations, thus saving the disease phenotypes by achieving prompt treatment[49]. Leber congenital amaurosis (LCA) is autosomal recessive retinopathy with both early and severe onset, causing severe visual loss due to premature transcription termination due to the presence of ~~CEP290 gene's~~ point mutations in the CEP290 gene at the intron branch point, followed by complete loss of pyramidal cell function in both eyes at birth or within one year of birth in infants[50, 51]. Many previous studies on LCA disclosed that the AAV5 (adeno-associated virus type 5) vector ~~delivered~~delivers Cas9 and CEP290-specific gRNAs to the retina by targeting the point mutation region to invert or delete it as a whole, thereby restoring the normal expression of CEP290 gene[52-54]. Although still in the early clinical trial stage, this recent approach is currently being used for treating type 10 congenital mongolism patients[55]. It has also been suggested that using mRNAs encoding nucleases, like CRISPR/Cas9 and gRNA, and their DNA editing property in target cells has the potential to be effective in Cystic fibrosis (CF) patients with the impact of potential mutations[56]. Many past literary insights reported that an adeno-associated virus vector might be used to express the CRISPR-associated protein 9 (Cas9) of *Campylobacter jejuni* with adenine deaminase activity as well as the corresponding sgRNA to achieve accurate correction of oncogenic mutations in the telomerase gene promoter region of glioma cells[57].

Additionally, numerous prior studies have also depicted that CRISPR/Cas9

technology also ~~reflected certain~~has application prospects in the treatment of hematological diseases: for example, when congenital G6PD (glucose-6-phosphatase-dehydrogenase) deficiency patients~~, ingested~~ ingest fava beans, acute hemolytic anemia~~, and~~ with several manifestations ~~occurred~~occur along with sickle cell anemia and β-thalassemia ~~that were~~ — a condition caused by mutations in the β-globin gene (HBB). Wu et al. effectively corrected G6PD and HBB point mutations by giving ~~Cas9-sgRNA and homologous donors~~composite injections into single-cell human embryos in Cas9-sgRNA and homologous donors [45, 58, 59], thereby demonstrating that CRISPR/Cas9 might also become a valuable therapeutic tool in human genetic diseases.

Application of CRISPR/Cas12 System in gene editing ~~disease~~ therapy

In 2015, Zetsche et al. characterized the Cas12a protein and identified two candidate enzymes from Eosinophilaceae and Lactobacillaceae, and demonstrated that Cas12a protein was able to conduct effective genome editing activity in human cells[21]. Unlike Cas9, the crRNA used by cas12a has only 42 nt[21], which offers many advantages in design and also facilitates delivery and simplification of the multiplex ~~gene~~gene-editing process. According to Verwaal, LbCas12a and FnCas12a show editing efficiency comparable to Cas9 in yeast cells and are expected to be good alternatives to Cas9 in the future. However, it is not widely used in practice， so there are very few instances of ~~genomic~~genome editing for disease treatments. Recently, some researchers engineered wild-type AsCas12a and designed a nuclease called enAsCas12a, which can show ~~high~~betterfold gene editing activity at the TTT PAM site, ~~and~~greatly improv~~ing~~e the editing efficiency of base C to base T. Peter et al. also applied enAsCas12a for genetic screening in human cells, and in the future, the CRISPR/Cas12 system might also play a larger role in disease

Commented [y1]: Revolution in Gene Medicine Therapy and Genome Surgery

Commented [y2]: CRISPR/Cpf1 enables fast and simple genome editing of Saccharomyces cerevisiae. Yeast.

Commented [y3]: Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing

Commented [y4]: Optimization of AsCas12a for combinatorial genetic screens in human cells

treatment.

Application of CRISPR/Cas13 System in gene editing ~~disease~~ therapy

On the contrary, the Cas13 protein family also ~~reflected good superiority~~seems to be promising in RNA knockout and editing, ~~which was in accordance with a study~~as reported by Cox et al. ~~in which~~In their study, catalytically inactive Cas13 (dcas13) was combined with ADAR2 to target transcripts to mammalian cells for editing of RNA bases A to I and correcting certain mutations in genetic diseases[27, 60]. Recently, Feng ~~Z~~et al. also developed a brand-new CRISPR/Cas gene-editing technology using dCas13, called RESCUE, in which ADAR2 enzyme targeted both base C in RNA and unwanted base C in tRNA and precisely modified them to base U, thereby achieving the purpose of changing the protein without modifying the DNA by altering the mRNA injunctions[61].

N[6]-methyladenosine (m[6]A) is a common post-transcriptional RNA methylation modification in eukaryote mRNA responsible for mRNA modifications, which sometimes in an abnormal state can trigger a series of diseases.[62] This was also confirmed by Liu et al. by using an m6A RNA editing tool constructed by phase fusion of dCas13 and gRNA and precisely edited m[6]A in the nucleus and cytoplasm. It could correct the methylase abnormality,[63] bringing the latest breakthroughs in RNA editing. Thus, CRISPR/Cas technology can provide broader research ideas and application prospects for the treatment of many diseases by precisely editing genes ~~by~~and rectifying the diversity of gene variants that cause diseases.

In recent years, CRISPR/Cas technology has emerged as a potentially powerful tool in cancer research and treatment~~, by~~. It offers genetic screening of oncogenic mutations and ~~altering~~can alter expressions of tumor suppressor genes.[64] ~~expressions, followed by~~

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It can be applied in CAR-T cell immunotherapy and immune checkpoint blocking therapy applications[46, 65, 66], thereby exploring and validating novel therapeutic targets in several preclinical studies involving tumors.

Application of CRISPR/Cas technology in multi-drug resistance analysis and treatment

CRISPR/Cas technology can be judiciously utilized to assess the surmounting multi-drug resistance (MDR) that has emerged as a serious public health threat due to inappropriate clinical antibiotic usage[67]. MDR usually occurs due to horizontal gene transfer (HGT) of antibiotic resistance genes (ARG) mediated by plasmids into other pathogenic bacterial forms resulting in frequent development of multidrug resistant strains[68]. Kelli et al. discovered a significant negative correlation between CRISPR/Cas loci in bacteria and detected the acquired antibiotic resistance by sequencing the *E. faecalis* genome.[69] thatIt was revealed that strains without CRISPR/Cas loci were more likely to acquire external resistance genes than strains with CRISPR/Cas loci[70].

It is now reported that nearlyNearly 26,000 Enterobacteriaceae infections are caused per year in China by ESBLs (extended-spectrum β-lactamases) producing *E. Coli bacteria each year in Chinacoli* [71]. Kim et al. developed a technique called Re-Sensitization to Antibiotics from Resistance (ReSAFR)[68], which encoded. In this technique, the CRISPR/Cas9 system phages to completefacilitates, the antimicrobials intracellular delivery of antimicrobials, followed by sgRNA-guided Cas9 specific cleavage of resistance-mediating genes present on the same plasmid as the target genes so that antibiotic-resistant cells become re-sensitive to antibiotics. As ReSAFR improves the practical value of the CRISPR/Cas9 system, it might become an effective approach to curb the formation of multidrug-resistant bacteria[68, 72]. Since in recent years, methicillin-

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resistant *Staphylococcus aureus* (MRSA) has become a major nosocomial pathogen worldwide, Kyeonghye et al. developed a simple, rapid, and highly sensitive method to detect MRSA, ~~named~~i.e., DNA-FISH[69] to rapidly and reliably detect MRSA and provide effective treatment ~~to patients on time by using~~. In this technique, dCas9/sgRNA complex is used as the targeting material and a nucleic acid stain SYBR Green I as the fluorescent probe to capture the MRSA DNA by specifically recognizing the mecA gene sequence with sgRNA~~, with~~. It offers a detection sensitivity of 10 CFU/mL, which is sufficient for ~~MRSA~~ effective detection of MRSA since the mecA gene, mainly present in resistant bacteria, is the prime underlying cause of MRSA resistance to β-lactam antibiotics~~, therefore~~. Therefore, MRSA can ~~be~~ promptly be identified based on the mecA gene for effective antimicrobial therapy[73]. Kotaro et al. also reported a positive outcome while developing the CRISPR-Cas13a-based antimicrobials capable of sequence-specific killing of MRSA, which is expected to be put into practical use as a therapeutic agent[74]. ~~Several~~Many of the emerging technologies mentioned above have a tremendous potential to combat some of our most critical clinical predicaments in world public health problems.

~~CHALLENGES FACING THE APPLICATION OF~~Challenges to the Application of CRISPR/~~CAS TECHNOLOGY~~Cas Technology

CRISPR/Cas technology is indeed a convenient, easy-to-use biomedical tool having a wide range of potential applications, but at the same time, there are certain unsolved problems in ~~the~~its application ~~of this technology~~ that should be accurately recognized.

Off-target effect

CRISPR has proven to be a highly versatile gene-editing tool with great potential in a wide

range of problems such as gene therapy, drug discovery, and gene modification in plant technology. However, the accuracy and reliability of CRISPR technology might be severely hampered by off-target effects because of unintended cleavage at untargeted genomic loci that do not match sgRNA, thus resulting in severe genomic aberrations[75].

When CRISPR technology is used for nucleic acid detection, the presence of an off-target effect can cause false positive or false negative results, affecting the accuracy of clinical diagnosis, and. The off-target effect is the main limiting factor affecting the application of CRISPR technology in clinical practice. In 2019, Joung et al. reported that the Cas9-based DNA editor experienced a severe off-target phenomenon while editing single bases and mutating a large number of unrelated DNA and RNA[76]. A correct interpretation of genomic data, along with the strategies for the detection of off-target mutations and minimizing off-target cleavage efficiency, are still some of the urgent problems that need to be addressed at present. For example, technologies such as GUIDE-seq[77], Digenome-seq[73], and CIRCLE-seq[78] have been widely used to detect off-target effects. In recent years, after an in-depth study of the factors affecting off-target effects, it has been found that the current strategies to solve off-target effects mainly include: (I) predicting off-target sites: Using CRISPOR, CHOPCHOP, and other tools, gRNAs can be designed online to analyze potential off-target sites, so that researchers can select gRNAs with low off-target effecteffects as much as possible. The recently introduced DISCOVER-Seq technology[79] can identify the exact site of CRISPR cutting genome, with simple processes and accurate results. In addition, the structure of sgRNAs and the activity of Cas protein are closely related to the off-target effecteffects. (II) Optimizing the design strategy of sgRNAs: Doench et al. found a significant reduction in off-target effects after

establishing new sgRNA design rules by optimizing the composition of sgRNAs. (III) Altering the structure of the Cas enzyme: Slaymaker et al. improved the binding rate to the target sequences using mutants of the Cas9 protein.

Safety and ethical issues

Recently, a study reported that a large number of unnecessary repetitive fragments appear when gene insertion is performed in mice using the CRISPR/Cas technology, which cannot be detected using the standard PCR analysis[80]. On the other hand, it was also proposed that in human embryonic cells, frequent DNA breakage can lead to the loss of the entire chromosome, or sometimes a large part of the chromosome, which can pose significant challenges for mutation corrections[81]. Another therapeutic intervention is the usage of Adenoadeno-associated virus (AAV) vectors that are often used with CRISPR/Cas9 for targeted gene- editing, which. They are sometimes unable to load larger genes, and might adversely affect the functioning of AAV vectors carrying exogenous DNA fragments inserted into human chromosomes due to insertional mutagenesis phenomenon. Henceforth, all combined efforts should be directed towards implementing the strong gene-editing abilities of CRISPR/Cas technology for the treatment of related diseases, while preventing any untoward genome-editing behaviors and similar negative events, as well as avoiding irreversible and unethical mutational consequences during treatment of such diseases..

Quantitative and high-throughput detection and sample pretreatment

Since CRISPR/Cas technology accurately conducts a quantitative analysis of target nucleic acids, it is deemed important for providing rapid and ultrasensitive data relevant

for prompt disease management and treatment, but. However, HOLMES and DETECTR, two detected CRISPR-Cas effectors, might not be capable of precise target quantification for Cas12-based detection platforms[30, 82]. In addition to that, multi-channel detection is also very crucial for distinguishing different pathogens as well as single-base polymorphisms for an accurate diagnosis, but only SHERLOCKv2 contains multi-channel detection ability at present[29], highlighting its innate potential as a rapid and quantitative detection platform.

With the more and more extensive application of CRISPR/Cas technology in clinical diagnosis, the sample sources, as well as the influencing factors within the samples, will be gradually expanded and diversified.[83] while Meanwhile, the complex sample pretreatment steps will also increase the probabilitypossibility of sample contamination, resulting in insufficiency and dilution oferroneous results. Feng Z et al. described a rapid detection protocol, Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON)), which has been developed to pair with SHERLOCK and SHERLOCKv2 for performing rapid chemical and thermal treatments on clinical samples to inactivate certain enzymes that degrade gene targets[84]. Therefore, it is very important to select a simple, efficient and economical sample pretreatment strategy for opening up novel avenues to tackle genetic diseases in a precise manner.

Despite significant technical improvements, the above-mentioned challenges should be further addressed for optimizing genomic stability in future studies.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As stated by Doudna et al., "The potential for CRISPR/Cas technology applications is enormous, affecting almost all aspects of life and providing inspiration for future

technological breakthroughs"[85],. CRISPR/Cas technology may revolutionize diagnostic and therapeutic research in clinical diseases and become a versatile tool in practice in the field of clinical medicine ~~soon~~ as ~~the~~a pathogen detection platform due to its high efficiency, portability, and low-cost factor, ~~and~~. Moreover, the developed diagnostic tools based on the CRISPR/Cas system are ~~extremely~~highly suitable for large-scale screening tasks in the frontline, community hospitals, and resource-limited environments[83], thereby initiating rapid and accurate detection ~~while~~and further promoting the development of point-of-care testing (POCT). Simultaneously, emerging CRISPR/Cas technology is being used with renewed efforts for discovering new therapeutic targets and detecting biomarkers to provide more accurate and scientific avenues for the early diagnosis and clinical treatment of oncological diseases[38, 65, 66]. Since the application of CRISPR/Cas technology in bacterial resistance detection has the advantages of simplicity, rapidity, and sensitivity, the recently developed platform can be more optimized by applying more detection methods for discovering various pathogens, which will be essential to prevent the spread of drug-resistant bacterial infections in hospitals[69, 74].

Owing to the fact that as CRISPR/Cas technology has an undeniable tremendous potential in clinical applications and scientific research technology, many ~~long~~long-term clinical studies are ~~the need of the hour~~required to elucidate biological mechanisms behind disease development and progression~~, making~~. This will make CRISPR/Cas technology an emerging discipline that provides better health care and improves human health more efficiently. It is also suggested that CRISPR will certainly have more exciting results in the future, bringing us unlimited possibilities;, thus, providing novel molecular therapies and ~~finally~~ promoting the development of the life sciences.

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Footnotes

Conflict-of-interest statement: Authors declare no conflict of interests for this article.
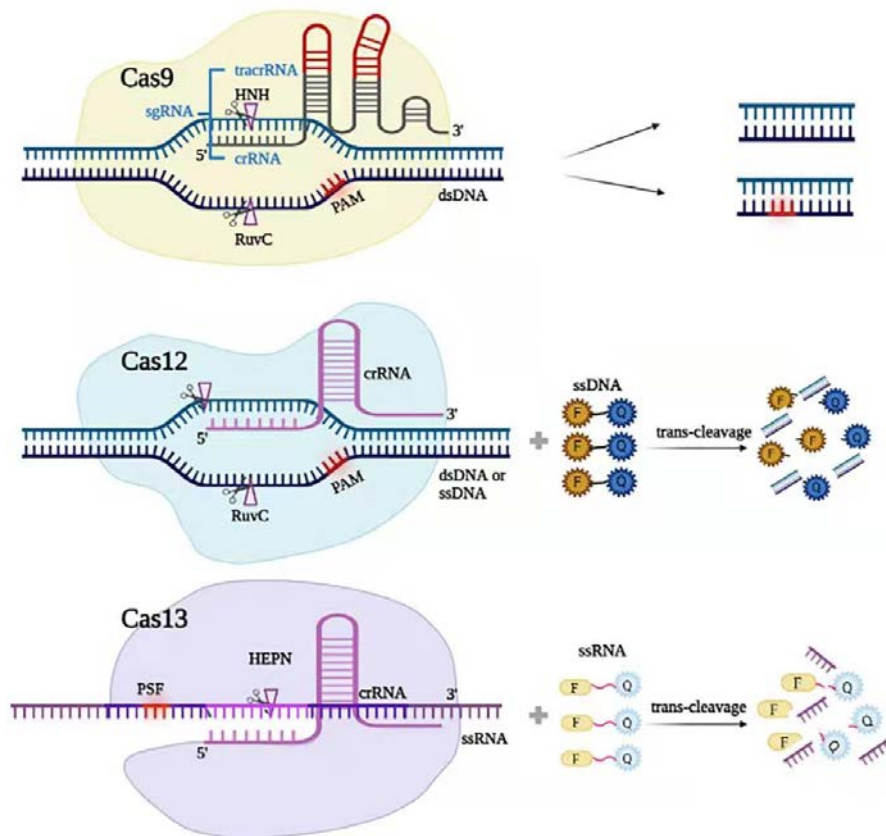
Figure 1. Collateral activity of Cas9, Cas12, and Cas13.

Table 1 Features of CRISPR/Cas System

CRISPR type		Specific cleavage	Collateral cleavage	Secondary messenger	CRISPR inhibitors	References
	I (Cas8)	DNA	Not identified to date	Not identified to date	Yes	[4]
Class 1	III (Cas10)	RNA	RNA, ssDNA	cOA (cyclic oligoadenylate)	Not identified to date	[6-8]
	IV (Csf1)	Not studied yet	Not studied yet	Not studied yet	Not studied yet	[16]
	II (Cas9)	DNA	Not identified to date	Not identified to date	Yes	[4-5] [16-20]
Class 2	V (Cas12, Cas14)	DNA	ssDNA	Not identified to date	Yes	[21-23]
	Ⅵ (Cas13)	RNA	RNA	Not identified to date	Not identified to date	[24-26]

Table 2 CRISPR-based pathogen nucleic acid detection system

Nucleic acid detection system	Cas	Target DNA	Amplification method	Test method	Time	Sensitivity	Specificity	References
SHERLOCK	Cas13a	Zika virus etc.	RPA	Fluorescence	2–5 h	2×10^{-8}M	High	[26]
SHERLOCKv2	Cas13a, Cas12a, Csm6	Zika virus, Gordon fever virus, yellow fever virus, etc.	RPA	Fluorescence Test strip	0.5–4 h	8×10^{-21}M	High	[29]
DETECTR	Cas12a	HPV	RPA	Fluorescence	1 h	10^{-18}M	High	[30]
STOP	Cas12b	2019-nCoV	LAMP	Fluorescence	1 h	100 copies	High	[31]
CADD	Cas9	HPV	—	Fluorescence	30 min	10^{-15}M	High	[32]
CARVER	Cas13a	Influenza A	RPA	Test strip	2 h	-	High	[33]
Smartphone testing	Cas13a	2019-nCoV	—	Fluorescence	15–30 min	10^{-4}M	High	[34]
Tandem nuclease	Cas13a+Csm6	2019-nCoV	—	Fluorescence	20 min	3.1×10^{-5}M	High	[35]

Table 3 CRISPR-based tumor diagnostic system

System	Cas	Tumor type	Sensitivity	Specificity	References
RCH	Cas9	NSCLC	10^{-15}M	High	[40]
CUT	Cas9	colorectal cancer	< 0.01%	High	[41]
SHERLOCK	Cas13a	NSCLC	0.1%	High	[26]
SHERLOCKv2	Cas13a, Cas12a, Csm6	NSCLC	8×10^{-21}M	High	[29]
DETECTR	Cas12a	HPV	10^{-18}M	High	[30]