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CRISPR/Cas System in Human Genetic Diseases

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Abstract:

Gene editing involves altering a particular sequence within an organism's genome using gene-editing techniques. Efficiently and accurately insert, delete or replace genes to alter their genetic information and phenotypic characteristics. DNA nuclease-based gene editing technology has advanced rapidly, from the first-generation editing system ZFNs, the second-generation TALENs to the third-generation CRISPR/Cas9 system, the efficiency of gene editing has been continuously improved, the cost has been gradually reduced, and the application scope has been expanding. The new classification system categorizes CRISPR-Cas proteins into Class I and II as the two main classes. Class I encompasses several subtypes, including Type I, III, and IV, all form complexes through the coordinated function of multiple Cas proteins. While Class II includes Type II, Type V, and Type VI, which all rely on single large Cas protein to perform all response. By designing single guide RNA (sgRNA), CRISPR can be used to target any gene sequence intended to be edited, achieving the wanted therapeutic outcome in treatment of inherited genetic disease. Although potential for off-target leads to undesired genetic alterations even result in oncogenesis, CRISPR-Cas still works as powerful therapy and gene editing tool in all aspects. Future research requires us to solve off-target issue. This review systematically introduces CRISPR-Cas systems as promising therapeutic strategy towards various genetic disease, explaining molecular mechanism and classification of CRISPR and differences among them.

Keywords: CRISPR/Cas system; gene-editing; human genetic diseases.

1. Introduction

As an effective tool for genome modification, CRISPR was first found in bacteria and archaea as part of adaptive immune system, allowing defending invasion by foreign genetic elements [1-3]. During the initial invasion, the immune system of microorganisms captures those foreign genetic fragments that are incorporated into the CRISPR array as additional spacers. Therefore, the unique CRISPR sequence record has been created with memory corresponds to past invaders. When similar invaders attempt to infect the cell again, the CRISPR-Cas system applies stored spacers which respond to prices cleave the invader DNA, thereby protecting the host cell from infection [2,3]. Those CRIPSR arrays are composed by repeated invaded DNA sequence, which can be transformed into CRISPR RNA (cRNA) with complementary sequence to target DNA. While another molecule called trans-activating CRISPR RNA (tracrRNA) which is a stretch loop binds to cRNA, forming a stable RNA complex. Such complex is crucial for proper function of CRISPR-Cas system because it ensures corrected folding of cRNA and becomes functional [3]. By designing and editing both cRNA and tracrRNA, their combination single guide RNA (sgRNA) can be used to aim at any gene designated for editing [1,2]. The initial phase of gene editing involves identifying the target DNA. As early mentioned, the specific designed sgRNA is introduced and binds with target DNA, while the tracrRNA provides a scaffold which allows Cas9 protein to attach to targeted part. The two regions which construct Cas-9 are recognition (REC) lobe and the nuclease (NUC) lobe [3]. PAM is a short-conserved sequence which is usually composed of three nucleotides—" 5'-NGG-3". Once the PAM is detected by Cas9, the recognition and binding of target DNA can be initiated [1]. However, except from the most typical "NGG" sequence, there are still many varieties exist. Researchers have been always trying to improve Cas9's PAM sites compatibility to optimize editing efficiency. Several Cas variants are developed to recognize multiple PAMs, like NG, GAA and GAT [2]. Once the protein bonds to target DNA at Pam site, the cleavage process begins. Two nuclease domains exist—HNH and RuvC domain responsible for cutting the DNA strand which complementary to the crRNA and non-complementary DNA strand respectively [1,2]. The cleavage takes place in three base pairs upstream of the

PAM sequence, resulting in a double-stranded break (DSB) [2]. Since PAM sequence does not appear in the bacterium's own array, PAM sequence plays the crucial role in genome editing efficiency and accuracy.

Once the double-strand break (DBS) occurs, the cellular DNA repair systems are activated. Two DNA repair pathways exist: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). NHEJ is the most common but error-prone mechanism, repairing DBS by directly ligating the broken DNA ends [1-3]. Without requiring homologous DNA template, mutation may occur by small insertion or deletion. As NHEJ might result in function loss of target gene, it works effectively in the purpose of gene knockout and inactivates target gene. On the other hand, HDR is an accurate repair process that relies on a homologous DNA template with sequences matching the DSB [2,3]. By introducing specific templet, the precise gene insertion or replacement can be achieved. Depending on the gene-editing objectives, either repair mechanism is chosen to determine the outcome.

2. Classification

Haft et al. developed the first classification of the CRISPR-Cas system, drawing from research on archaeal and bacterial genomes [4]. This system comes from the prokaryotic adaptive immune response, which targets and destroys invading genetic material by degrading DNA and RNA. It is a complex system capable of functioning independently in unicellular organisms. According to the early bioinformatic analysis, four highly conserved Cas genes were defined as Cas1, Cas2, Cas3, and Cas4. Further research divides this system into more families according to various criteria, Cas5 and Cas6 were introduced as the new part. Cas1 is considered as the basis for the classification criteria [4].

Eight Cas subtypes were categorized and named based on their host species . For example, CRISPR system Ecoli are subtypes only found in Ecoli labeled as cse1, cse2, cse3, cse4, while CRISPR system Apern refers subtypes found in in Aeropyrum pernix as csa1, csa2, csa3. Therefore, the subtypes were designated as Apern, Nmeni, Tneap, Dvulg, E. coli and Hmari, corresponding to Aeropyrum pernix, Neisseria meningitidis serogroup A strain Z2491, Thermotoga neapolitana DSM 4359, Desulfovibrio vulgaris strain, Escherichia coli (E. coli) and Haloarcula marismortui strain ATCC 43049 [4].

The updated classification system divides Cas proteins into two main Classes. Class I consists of subtypes such as Type I, III, and IV, all of which form complexes through the combined activity of multiple Cas proteins. While Class II encompasses Type II, V, and VI, relying on an individual Sizable Cas protein to carry out the entire response [4].

2.1 Class I Systems

Class I systems consist of Type I, Type III, and Type IV which emphasize collaboration of multi-Cas proteins. The Type I system has Cas3 as signature protein which works with Cas1 and Cas2 to perform helicase and nuclease activities and spacer sequence integrated [4]. The Type I system also transcribes Cas5 to 7, participating in pre-crRNA processing. This system is additionally subdivided into six different variants, ranging from IA to IF, each with distinct operon configurations. For instance, Type IA is marked by the signature genes Cas8a2 and Cas5, while Cas8b and Cas8c distinguish Type IB and Type IC, respectively [4].

Type III system has the signature protein Cas10 which has a palm domain for catalytic activity and a zinc-binding helical domain. Instead of including Cas1 and Cas2, Type III uses crRNA originating from Type I or II CRISPR sequences [3,4]. Cas10 protein also has great varieties among Type III subtypes include III-A, III-B, III-C, III-D, III-E, and III-F. For example, Type III-A and III-D are capable of DNA cleavage due to the presence of Cas1, Cas2, and Cas6, while Type III-B primarily interacts with RNA and lacks those genes.

Type VI are the most distinct within Class II, which has the signature protein C2c2 that include unique HEPN domains [2,4]. HEPN domains allow Type VI systems specific cleave RNA instead of DNA. Type VI systems are subdivided into VI-A, VI-B, and VI-C, which differ in the placement of HEPN domains and catalytic motifs across the subtypes. For instance, VI-A includes the signature genes DinGas or csm4, whereas Type VI-B lacks genes [4].

2.2 Class II Systems

In contrast to Class I systems, Class II Systems emphasizes the sole Cas protein effect [1,3,4]. For type II systems, Cas9 is the signature protein which performs all immune response steps including recognition, sequence integration and cleavage [3,4]. Cas9 protein has been widely used in genetic therapy or scanning technology. Due to the capability of directly applying modification of tracrRNA, Cas9 plays a crucial role in both DNA target and crRNA processing.

Type V systems feature the signature protein Cpf1 (Cas12a), which resembles Cas9 but does not contain the HNH domain. It forms a uni-subunit crRNA complex and uses different PAM sequences. It also has several subtypes including V-A, V-B, and V-With differences in effector proteins' structure and function [4].

Type VI systems are the most distinct within Class II because they have HEPN domains in their signature protein

C2c2 [3]. Like previously mentioned, the presence of HEPN domains allow Type VI specifically cleave RNA rather than DNA. Type VI systems are further categorized to VI-A, VI-B, and VI-C with variations in HEPN domain's location and catalytic motifs across subtypes [4].

3. Molecular Mechanism of CRISPR-Cas System

3.1 Adaptation

The adaptation serves as a cornerstone in CRISPR-Cas Systems by proving genetic memory which ensures the effective combat of re-invading foreign genetic material. As noted in the introduction regarding the recognition step, during the initial invasion, new spacer sequences are integrated into the CRISPR sequence, accompanied by the production of a new repeat sequence. However, the detailed molecular mechanisms remain unclear [3]. The key step includes selecting protospacer material from the target DNA and integrating the spacer into the CRISPR sequence. Cas1 and Cas 2 are key proteins to spacer integration. During the process, Cas1 and Cas2 interact and form dimer complex, facilitates spacer material acquisition into array [3,4]. Additional factors such as tracrRNA, Cas9, Csn2 in Type II-A, and Cas4 in Type I-B are also required for spacer acquisition, although their specific functions remain unclear [3,4]. But various experiments have validated that Spacer acquisition indeed occur across several CRISPR-Cas variants, like Type I-A in Sulfolobus, Type I-B in Haloarcula hispanica and Type I-E in E,coli [3]. The PAM is critical for spacer acquisition since it determines the location of resulted DSB and prevents self-targeting [1]. Although the mechanism of exact process of protospacer remains unknown, the newly acquired spacers are found near to a PAM sequence. In different Cas Type systems, Cascade complex enhances accuracy of the PAM recognition, like Cas1 and Cas2 in Type I-E system [4].

The leader sequence is positioned at CRISPR array's start, playing an essential role in spacer acquisition. It serves both as the location for incorporating new spacers and as a template for generating new repeat sequences. The orientation and polarity of spacer integration are determined by the leader sequence, and its palindromic nature facilitates the formation of DNA structures that attract the Cas1-Cas2 complex for spacer integration [4].

3.2 Expression

The expression process in CRISPR-Cas systems is producing RNA-protein guide complex (crRNP) that can recognize and cleaving foreign DNA by CRISPR loci transcriptions. While such processes remained similar across organisms, there are still type-dependent differences. All systems have the same process of initial transcription of CRISPR loci, but some species such as E. coli, transcription of CRISPR begins in the leader sequence region [4]. The primary transcript in the CRISPR-Cas system is called pre-crRNA which is a precursor lengthy RNA molecule. In the processing stage, pre-crRNA is cut into functional units, each matching a single spacer sequence. The Cas protein involved in this process varies based on the type of CRISPR system [1-3]. Most importantly, the three coexist CRISPR-Cas types only process their own pre-crRNA.

Type I and III systems have many common points in processing of pre-crRNA and structure of formed crRNP complex. They all use Cas6 protein to Operate pre-crRNA except for the Type I-C uses Cas5d protein [3,4]. For the E. coli Cascade complex, Cas6e and crRNA serve as main component, which also contain one Cse1, two Cse2, one Cas5e, and six Cas7 proteins. In Type III systems, the pre-crRNA is processed by Cas6, followed by ruler-based trimming 3' end to produce mature crRNA. While the study shows that Cascade complex can be formed in cells that are devoid of crRNA and later be loaded with crRNA independently, crRNA processing and Cascade assembly can be two separate processes [4]. Although Type II systems also need the Cas9 protein in pre-crRNA processing step, it crRNA biogenesis mechanism relies on the host's RNase III and a trans-encoded small RNA (tracrRNA) which pairs with the pre-crRNA, which is distinct from other two type system [3,4].

3.3 Interference

The last stage of adaptive immune response is Interference. The Complete crRNA produced in expression step acts as guide RNA, binds to Cas protein and recognizes protospacer sequence on the target DNA, eventually triggering the target degradation. This step is also system subtypes varies [1,3,4]. Compared to the Class-II systems only need an individual Cas protein for target interference, Class-I systems require the CASCADE complex to execute the last step of defense. Class-I systems rely on the CASCADE complex to carry out the final defense step, whereas Class-II systems only need an individual Cas protein for interference [4]. Systems of Type I, II, and V generally detect PAM regions to locate the target DNA, whereas Type III systems employ a 5′ marker to differentiate self from non-self, thereby avoiding self-directed attacks [4].

In Type I systems, the stage engages both the CASCADE complex and the Cas3 effector protein. Via the Cse1 large subunit, CASCADE complex that directed by crRNA identifies the PAM site [2-4]. During the recognition, the CASCADE complex binds to target DNA, forming

R-loop by annelation with the protospacer region of crRNA. Therefore, the shape transformation is created in CASCADE complex and eventually triggers cleavage performed by Cas3 which subsequently unravels the RNA-DNA hybrid with the 3′-5′ orientation, using its nuclease domain to cut the target DNA [4].

In Type II systems, cleavage process is performed by Cas9, which is guided by the DBS tracrRNA duplex, leading to a DBS in the target region. Compared to Type III, the binding of the Csm/Csr structure with complementary region of the target gene activates the Cas10-Csm, Cas7 proteins , and Cas7 proteins, which cleave the target via RNA-activated DNases. Type V operates similarly to Type II, except for Type V-A, which only requires crRNA for target cleavage [3,4].

4. Application of CRISPR-Cas system in Genetic Diseases

4.1 Inherited eye Disease

The CRISPR-Cas9 system is capable of offering potential for both therapeutic intervention and deeper understanding of ocular pathologies. Eye-related disorders like retinitis pigmentosa, glaucoma, Leber's congenital amaurosis glaucoma, and congenital cataracts have been the focus of recent CRISPR-based research, showcasing the system's capability to accurately identify and alter genetic mutations responsible for diseases.

In the case of retinitis pigmentosa (RP), a hereditary retinal dystrophy, CRISPR-Cas9 has been utilized to fix specific gene mutations responsible for the disease, such as those in the RHO gene [5-7]. By delivering guide RNA and Cas9 components directly to the retina, researchers have demonstrated that it is possible to halt or even reverse the degeneration of photoreceptors, which are critical for vision [5-8]. For instance, in a study, the CRISPR-Cas9 editing technique successfully corrected the Rho (S334) gene through subretinal injection of the gRNA-Cas9 plasmid, improving visual activity by preventing retinal degeneration in rats [5]. This gene-editing approach not only preserves retinal structure but also improves visual function in animal models, indicating significant potential for treating RP in humans.

For glaucoma, particularly primary open-angle glaucoma (POAG), CRISPR-Cas9 has shown promise in targeting the myocilin (MYOC) gene, mutations of which are known to contribute to the disease by causing abnormal protein accumulation that increases intraocular pressure (IOP) [5-7]. By disrupting the mutant MYOC gene in experimental models, CRISPR-Cas9 has successfully reduced IOP and prevented further damage to retinal ganglion cells, offering a potential one-time treatment for this otherwise chronic and progressively debilitating condition [5-7].

Congenital cataracts, a major cause of childhood blindness, have also been a target for CRISPR-based interventions [7,8]. Researchers have achieved notable progress in employing CRISPR-Cas9 to fix genetic mutations that cause congenital cataracts, specifically targeting the CRYGC and GJA8 genes. In one study, Cas9 mRNA and guide RNA (sgRNA) were precisely injected into the cytoplasm of mouse zygotes. This process facilitated the correction of a one-base-pair deletion mutation in the CRYGC gene, which would typically halt protein synthesis early, leading to cataract formation [7]. The corrected zygotes developed normally, with 24 out of 78 mice showing successful mutation correction. Similarly, in rabbits, the co-injection of Cas9 mRNA and sgRNA targeted the GJA8 gene [8], crucial for lens transparency, achieving nearly 100% mutation correction. These experiments demonstrate the effectiveness of CRISPR-Cas9 in potentially preventing congenital cataracts by early genetic intervention.

Leber's congenital amaurosis (LCA), a severe retinal dystrophy that causes early-onset blindness, has been a significant focus of CRISPR-Cas9 research due to its genetic complexity [5,7]. Studies have targeted mutations in genes like KCNJ13 and RPE65, which are critical to retinal health and function [5-7]. For instance, Zhong et al. utilized Cas9 to modify the KCNJ13 gene in mouse zygotes, creating a model that mimicked human LCA [5]. This gene editing led to the preservation of retinal pigment epithelium (RPE) cells and prevented photoreceptor degeneration, a hallmark of LCA, thereby demonstrating the potential of CRISPR to slow disease progression.

Similarly, Jo et al. utilized Cas9 to fix a disease-inducing mutation in the RPE65 gene in a mouse model [5,7]. This intervention restored retinal function and provided a blueprint for potential therapeutic strategies aimed at human LCA cases [5,7]. The success of these animal studies has been encouraging, as they not only correct genetic mutations but also significantly restore vision, highlighting CRISPR-Cas9's promise in treating LCA and similar genetic retinal disorders.

However, the application pf CRISPR-Cas9 in treating eye diseases is not without risks. The potential for off-target mutations, where unintended location in the gene is edited, could lead to undesired genetic alterations, which may result in fatal consequences, including oncogenesis [5-8].

To mitigate these risks, various strategies are being explored. For instance, the Cas9 delivery method and sgRNA as ribonucleoprotein complexes (RNPs) offer a

transient expression of the editing machinery, reducing the likelihood of off-target effects and genotoxicity [7]. Furthermore, drug-inducible systems and self-destructing CRISPR constructs are being developed to more controllable and temporary activity of the Cas9 enzyme, enhancing safety profiles [8]. Moreover, researchers are investigating the use of base/prime editors, offering more precise gene editing capabilities without introducing DBS, thereby lowering the likelihood of unintended genetic alterations [6,7]

4.2 Hemoglobinopathies

Hemoglobinopathies is one of the most common inherited genetic disorders in the word, particularly as Sickle cell disease (SCD) and β-thalassemia. The mutation of β-globin gene (HBB) leads to defective hemoglobin production and severe clinical manifestation. However, CRISPR-Cas9 offers a promising solution for addressing complex genetic failure of hemoglobinopathies in three extend. Disease results from a single point mutation in the HBB gene, leading to the production of abnormal hemoglobin S (HbS) which causes red blood cells to become sickle-shaped and rigid Sickle cell in low oxygen conditions, eventually leading to the symptom like chronic pain, and organ damage and even death [9]. The first gene editing strategy for CRISPR-Cas9 is doing the direct correction of the HBB point mutation. Utilizing gRNA that targets the mutated region of the HBB gene in hematopoietic stem and progenitor cells (HSPCs), the Cas9 enzyme is able to induce a DSB [10]. In the HDR, as the synthetic DNA template with correct sequence is introduced and repair the cut, SCD can be potentially permanently cured by formation of healthy hemoglobin. Clinical trials have shown promising outcomes with a significant improvement in healthy hemoglobin production and an associated reduction in disease symptoms [10-12].

Another application of CRISPR-Cas9 in hemoglobinopathies is induction of fetal hemoglobin (HbF) expression. In the study, Researchers employed CRISPR-Cas9 to target and disrupt the BCL11A gene, a crucial regulator of HbF expression. Due to the composition of two gamma globin chains ($\alpha 2\gamma 2$), HbF can functionally replace the deficient in β-thalassemia or SCD. Therefore, by knocking out BCL11A the increasing level of HbF compensates for the hemoglobin failure disorder [12]. The simplicity and effectiveness of this such strategy have made it a central focus in current research and clinical trials.

In β-thalassemia, the absence of β-globin chain production results in excess unpaired α-globin, leading to an imbalance that forms unstable α-globin tetramers, which precipitate in red blood cells [10,12]. One innovative approach using CRISPR-Cas9 is to reduce the production of α-globin is, by targeting and downregulating HBA2 gene, normal β-globin production level can be restored. According to the research, by repairing HBA2 gene break targeted by CRISPR-Cas9 by NHEJ, HBA2 expression is reduced significantly, alleviating the imbalance with two globin chains and eventually improved blood function [10,11]. However, the main challenge remains in effectively delivering Cas9 components to the recipient cells. As the most widely used way to deliver genetic material, adeno-associated viruses (AAVs) still have limitations [12]. For example, such vector might be recognized by immune system which make adverse immune response. To address such issues, non-viral delivery methods, such as lipid nanoparticles and electroporation are being invented. lipid nanoparticles encapsulate CRISPR-Cas9 in the lipid membrane bag which can fuse with the cell, making the delivery process safer without concerning the associated risk of viral vector.

4.3 Muscular genetic disease

Duchenne muscular dystrophy (DMD), facioscapulohumeral muscular dystrophy (FSHD), and limb-girdle muscular dystrophy (LGMD) are three genetic muscle diseases caused by alterations in genes vital for muscle function, which leads to the muscle degeneration and disorder.

DMD is among the most frequently occurring forms of muscular dystrophy, arising from mutations in the dystrophin gene found on the X chromosome. An out-of-frame mutation in the open reading frame (ORF) leads to the production of a non-functional protein [12]. Nowadays, in the CRISPR-Cas9 therapy which involved exon skipping, utilize it to create a single cut near exon-intron junction. As DNA is repaired through NHEJ, the small insertions or deletions (INDELs) may be introduced, normal splicing signal can be interrupted and causing mRNA splicing to skip the mutated exon [2,3,12]. Therefore, the partially functional protein can still be produced, converting severe DMD phenotype into a milder Becker muscular dystrophy (BMD) phenotype.

Another approach is exon reframing, and it is also achieved by creating a single cut near exon-intron junction. Just like mentioned before, the INDELs in the genome change the whole ORF. However, those changes induce the frameshift, realigning the reading frame and converting to the functional one. By restoring the function of reading frame, functional protein can be produced and allow partial recovery. Both approaches have demonstrated encouraging outcomes in preclinical studies, including human induced pluripotent stem cells (iPSCs) and animal models like mice and dogs [12].

Facioscapulohumeral muscular dystrophy (FSHD) is mainly triggered by the shortening of the D4Z4 region on

the chromosome, resulting in the atypical activation of the DUX4 gene and its transcription factor, which ultimately causes muscle atrophy. However, study of CRISPR application in the FSHD field is still at a very early stage, particularly in DUX4-related animal models. To reduce expression of full-length DUX4 mRNA, researchers fused deactivated Cas9 (dCas9) with KRAB to repress DUX4 gene expression which reduce gene mis regulation caused by FSHD [12]. However, such therapy does not permanently change gene structure, sustained and repeated therapy is required to reach efficacy. Future research is still required.

Limb-girdle muscular dystrophy (LGMD) indicates collection of muscle disorders resulting from various gene mutations. As it involves multiple subtypes, targeted therapeutic approaches are required. LGMD2B is caused by dysferlin gene (DYSF) mutation while LGMD2D is caused by α-sarcoglycan gene (SGCA) mutation, Cas system is applied to induce homology-directed repair (HDR) to correct two causative gene mutation [12]. Such method shows the positive result when applied in induced pluripotent stem cells (iPSCs), however, in postmitotic skeletal muscle HDR does not reach the expected level. Researchers are exploring the new gene exploring method such as base editors (BEs) and prime editors (PEs) [11,12]. Although the CRISPR system indeed shows promising results in LGMD treatment, further study is required to improve therapeutic efficacy.

5. Conclusion

Despite CRISPR's promising applications in various fields, there are still many risks. The potential for off-target mutations, where unintended sites in the genome are edited, poses a significant challenge. Such off-target effects may result in undesired genetic variations, which may result in harmful consequences, including oncogenesis. Additionally, the potential for immunogenicity of CRISPR elements, especially those originating from bacterial sources, poses concerns regarding immune reactions that may reduce the therapy's efficacy or lead to inflammation and tissue injury. To mitigate these risks, various strategies are being explored. The delivery of Cas9 and sgRNA as ribonucleoprotein complexes (RNPs) offers a transient expression of the editing machinery, lowering the likelihood of off-target effects and long-term genotoxicity. Additionally, drug-responsive systems and self-deactivating CRISPR constructs are being created to provide more regulated and temporary Cas9 enzyme activity, improving safety measures. Moreover, researchers are investigating the use of base editors and prime editors, which offer more precise gene editing capabilities without introducing DBS, thereby lowering the risk of unintended genetic alterations.

References

[1] Asmamaw M, Zawdie B. Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. Biologics. 2021 Aug 21; 15:353-361.

[2] Xu Y, Li Z. CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. Comput Struct Biotechnol J. 2020 Sep 8; 18:2401-2415.

[3] Devashish Rath, Lina Amlinger, Archana Rath, Magnus Lundgren, The CRISPR-Cas immune system: Biology, mechanisms and applications, Biochimie, Volume 117, 2015, Pages 119-128, ISSN 0300-9084.

[4] Simran Bhatia, Pooja, Sudesh Kumar Yadav, CRISPR-Cas for genome editing: Classification, mechanism, designing and applications, International Journal of Biological Macromolecules, Volume 238, 2023, 124054, ISSN 0141-8130.

[5] undaresan, Y., Yacoub, S., Kodati, B., Amankwa, C.E., Raola, A. and Zode, G. (2023), Therapeutic applications of CRISPR/Cas9 gene editing technology for the treatment of ocular diseases. FEBS J, 290: 5248-5269.

[6] Ahmad I. CRISPR/Cas9-A Promising Therapeutic Tool to Cure Blindness: Current Scenario and Future Prospects. Int J Mol Sci. 2022 Sep 29;23(19):11482.

[7] Hu X, Zhang B, Li X, Li M, Wang Y, Dan H, Zhou J, Wei Y, Ge K, Li P, Song Z. The application and progression of CRISPR/Cas9 technology in ophthalmological diseases. Eye (Lond). 2023 Mar;37(4):607-617.

[8] Sharma G, Sharma AR, Bhattacharya M, Lee SS, Chakraborty C. CRISPR-Cas9: A Preclinical and Clinical Perspective for the Treatment of Human Diseases. Mol Ther. 2021 Feb 3;29(2):571-586.

[9] Hu X, Zhang B, Li X, Li M, Wang Y, Dan H, Zhou J, Wei Y, Ge K, Li P, Song Z. The application and progression of CRISPR/Cas9 technology in ophthalmological diseases. Eye (Lond). 2023 Mar;37(4):607-617.

[10] Sundaresan Y, Yacoub S, Kodati B, Amankwa CE, Raola A, Zode G. Therapeutic applications of CRISPR/Cas9 gene editing technology for the treatment of ocular diseases. FEBS J. 2023 Nov;290(22):5248-5269.

[11] Sharma G, Sharma AR, Bhattacharya M, Lee SS, Chakraborty C. CRISPR-Cas9: A Preclinical and Clinical Perspective for the Treatment of Human Diseases. Mol Ther. 2021 Feb 3;29(2):571-586.

[12] Min YL, Bassel-Duby R, Olson EN. CRISPR Correction of Duchenne Muscular Dystrophy. Annu Rev Med. 2019 Jan 27;70:239-255.