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Review

Noncoding RNA-targeted treatment for schizophrenia: CRISPR/CAS9

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ABSTRACT

Schizophrenia is a genetically related mental disorder in which most genetic changes occur in non-coding regions of the human genome. In the past decade, an increasing number of non-coding regulatory RNAs (ncRNAs), including microRNA (miRNA) and long non-coding RNAs (lncRNAs), have been strongly associated with schizophrenia. However, understanding the workings of ncRNA and genetic mutations in the pathophysiology of schizophrenia has failed due to insufficient technology and lack of appropriate animal models to effectively manipulate ncRNA genes. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9; CRISPR/Cas9) has been developed to enable researchers to overcome these challenges. This review article mainly focuses on the use of CRISPR/Cas9 editing of these regions to demonstrate the causal relationship between mutations in non-coding regions of genomic DNA that express schizophrenia-related ncRNAs and the pathophysiology of schizophrenia. Furthermore, although CRISPR/Cas9 technology is still in its infancy and immature for use in the treatment of diseases, its potential to transform this advanced technology into a clinical treatment for schizophrenia will be discussed. This review describes the application of powerful and viable CRISPR/Cas9 technology to manipulate ncRNA genes associated with schizophrenia.

Keywords: CRISPR/Cas9, gene editing, lncRNA, miRNA, Non-coding RNA, schizophrenia.

Schizophrenia is a severe mental disorder associated with neurodevelopmental abnormality in which patients exhibit both mental and behavioral impairment.[1] Most scientific evidence has shown that approximately 80% of schizophrenia cases are genetically transmitted.[2] Decades of research have shown that the interaction of genes and environmental factors greatly contributes to schizophrenia.[3] It has been found that the majority of genetic changes occur in non-protein-coding regulatory RNA (ncRNA), particularly micro RNA (miRNA) and long non-coding RNAs (lncRNA). miRNA and lncRNA are two regulatory ncRNAs that do not code proteins and differ in function, location, and size. miRNAs play important roles in post-transcriptional destabilization of messenger RNA (mRNA), translational suppression, or regulation of both molecular mechanisms.[4,5] lncRNAs are defined as non-protein-coding regulatory ncRNAs longer than 200 nucleotides (nt) in length. Although the function of lncRNAs is not as well understood as the function of miRNAs, recent studies have provided a better understanding of their role and have shown an association with psychiatric disorders such as autism, bipolar disorder, and major depression, including schizophrenia.[6-10] Since miRNAs and lncRNAs are highly expressed in brain tissue because they regulate genes necessary to maintain brain development and function, as abnormal brain development and maturation has been proven to be linked to schizophrenia. For example, manipulation of schizophrenia-associated

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miRNAs such as miR-132/miR-121 and miR-219 has demonstrated changes in neuronal activity and brain plasticity at synapses.[11-13] Further studies have revealed that these miRNAs directly regulate the synthesis of proteins required for synaptic plasticity or interact with factors likely to regulate permanent neuroplastic changes.[13,14] These recent findings of schizophrenia-associated miRNAs suggest that dysregulation of miRNAs and targeted genes is crucial for our understanding of the underlying biological causes of schizophrenia. Considering the multitude of miRNA alterations and their broad impact on target genes in schizophrenia, schizophrenia-associated miRNAs may be significant in the pathogenesis of schizophrenia. In past years, an increasing number of schizophrenia-associated ncRNAs such as miR-137, lncRNA Gomafu, etc. have been identified, and genetic changes in these ncRNA genes have strengthened the implications for the pathogenesis of schizophrenia.[8,15-17] However, the functional roles of these genetic variations in the development and progression of schizophrenia are not yet well understood. In fact, the functioning of these ncRNAs in schizophrenia development and the correction of genetic changes in genomic DNA that are key to developing new treatments for schizophrenia and the effective manipulation of these ncRNA genes have not been realized due to insufficient technology. In light of new developments, it will be possible to create or correct mutations in animal models using new genetic tools in genomic DNA, including coding and non-coding regions in schizophrenia, which will be the key to developing new treatments for schizophrenia. In recent years, a large number of miRNAs and lncRNAs associated with schizophrenia have been identified and characterized, thanks to a range of advanced technologies, including next-generation sequencing, high-resolution microarray, and genotyping. Understanding the genetic variations of ncRNAs in the development of schizophrenia has benefited from advanced technology and new tools from biology and other fields of science. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) (CRISPR/Cas9) is a recently developed revolutionary gene editing technology that can effectively manipulate non-coding regions of genomic DNA in human cell lines and animal models.

THE ROLE OF NON-CODING RNAs IN SCHIZOPHRENIA

The role of complex genetic components in the etiology of schizophrenia has been strongly proven by a large quantity of evidence. Indeed, the heritability of schizophrenia is approximately 80%.[2] Thus, new genomic tools offer hope for our understanding of the profound etiology of this complex genetically related psychiatric disorder. Genome-wide association studies have revealed a number of strong susceptibility loci for schizophrenia, most of which are located in non-coding regions of the genome for transcription of miRNAs and lncRNAs. Numerous studies have shown that most miRNAs and lncRNAs are highly expressed in the brain and that abnormal expressions due to genetic mutations and effects on target gene levels cause defective development, resulting in suppression of synaptic activity and mGluR (Metabotropic glutamate receptor)-dependent synaptic transmission. Since this affects the plasticity of the hippocampus, it may help us understand the pathogenesis of schizophrenia, among other neurological disorders.[18-24] In recent years, comparing the expression of different levels of miRNAs involved in schizophrenia with the help of high-throughput microarray techniques compared to control groups has identified and better elucidated a large number of dysregulated miRNAs in schizophrenia.[25,26] A genome-wide association study in a large-scale population revealed non-coding genes associated with schizophrenia, including miRNA genes and prominently miR-137.[16,17] The discovery of loci associated with schizophrenia has received great attention in this field, and the gene for a small ncRNA, miR-137, is located at locus 1p21.3. A large-scale genome-wide association study conducted by Franke et al.[27] included more than 40,000 participants and found that rs1625579 polymorphism in the miR-137 gene was strongly associated with schizophrenia.[17] To date, many in-depth studies have supported the association between miR-137 and schizophrenia and variant rs1625579 of the miR-137 gene as an indicator of early onset of psychosis in schizophrenia.[28] In a cohort study of 1,430

schizophrenia patients compared to 1,570 healthy individuals in a Chinese Han population, analysis of rs1625579, a single nucleotide polymorphism of the miR-137 gene was conducted; rs1625579 showed significant differences in allele frequencies between schizophrenia subjects and healthy control subjects,[29] while the single-nucleotide polymorphism rs66642155 allelic variant was positively correlated with the age of onset of schizophrenia and the degree of symptoms.[30] The molecular mechanism by which genetic variants of miR-137 present an increased risk of schizophrenia has also been associated with reduced fronto-striatal white and gray matter integrity, but no change in brain plasticity,[31,32] and may cause symptoms of poor concentration, low processing speed, cognitive impairment, etc.[33] In a recent detailed study of the zebrafish model by Giacomotto et al.,[34] it was demonstrated that suppression of miR-137, possibly responsible for the behavioral phenotype and associated with schizophrenia, resulted in impairment in synaptic functions and behavior in the neural network.

Apart from miRNAs, lncRNAs have emerged as one of the most important classes of ncRNAs in the regulation of gene expression, and it has been stated that changes and dysregulation of lncRNAs play an important role in the pathogenesis of various diseases. Only a few lncRNAs associated with schizophrenia have been identified, unlike miRNA associated with the large number of studied schizophrenia cases. Among them, Myocardial Infarction Associated Transcript (MIAT) also known as RNCR2 (retinal non-coding RNA 2), is the most recently discovered lncRNA and was strongly associated with schizophrenia. MIAT was first described by Blackshaw et al.[35] as a new member of the lncRNA family and was found to play a role in the regulation of differentiation of retinal cells in the embryo and associated with myocardial infarction.[36-38] MIAT is abundantly expressed in the nuclei of neurons during adult development. This lncRNA is also referred to as lncRNA Gomafu,[15] a Japanese word that reflects its spotted pattern in the nucleoplasm. Despite the 22q12.1 locus documented to be associated with schizophrenia and its expression in the nervous system, the link between this lncRNA and schizophrenia was disclosed in 2014.[8] Barry et al.[8] first demonstrated the strong correlation between MIAT and schizophrenia and elucidated the mechanisms, including downregulation of Gomafu in patients with schizophrenia: Rapidly decreasing Gomafu levels in response to neuronal activation and Gomafu-mediated impaired alternative splicing directly binds to two splicing factors, QK1 and serine/arginine-rich splicing factor 1, ultimately leading to abnormal regulation of two schizophrenia genes DISC-1 and ErbB4,[4,38] resulting in reduced activity of parvalbumin interneurons.[39] Shortly after identifying the association between lncRNA Gomafu and schizophrenia, its genetic alterations were subsequently reported to be associated with schizophrenia in different populations.[10] An analysis of genetic variants of lncRNA Gomafu in 1,255 cases diagnosed with paranoid schizophrenia compared to 1,209 healthy individuals in the Chinese Han population found that rs1894720 polymorphism was significantly associated with paranoid schizophrenia.[10] Aside from lncRNA Gomafu, there are other lncRNAs associated with mental disorders such as Evf2, BDNF-AS, and DISC-2, which have been previously reviewed and found to be associated with schizophrenia.[9]

CRISPR/CAS9 AND METHODS

Originally well described as an adaptive immune defense mechanism in bacteria, the CRISPR/Cas9 system is an emerging revolutionary and viable method for precise genome editing of a variety of organisms, including plants, animals, and even humans. With this method, genomic DNA stretches can be edited easily and precisely.[40-46] The CRISPR/Cas9 system usually consists of two components: Cas9 protein and guide RNA (gRNA). The Cas9 protein guided by gRNA is recruited to the target site and can cleave the genomic DNA at a specific site. The past years have witnessed the emergence of this innovative technology and dramatic advances in genomic DNA editing.[47] CRISPR/Cas9 technology can be used to manipulate genomic DNA elements targeting not only coding regions but also non-coding; these elements include small and long ncRNAs such as miRNAs and lncRNAs. Editing genomic DNA elements targeting non-coding regions is particularly important because silencing these ncRNA genes with

current, conventional RNA interference (RNAi) technology often fails due to their resistance to RNAi techniques.[47-50] Recently, a modified CRISPR/Cas9 method known as Double Excision CRISPR Knockout (DECKO) is applied, in which a lentiviral vector expressing two gRNAs simultaneously is used for manipulating and editing genomic DNA fragments from 100 to 3,000 base pairs (bp) in length; many obstacles have been overcome with the application of the method.[51,52] To date, a number of ncRNA genes in genomic DNA have been successfully silenced, including miRNAs (miR-21, miR-29a) and lncRNAs (UCA1, MALAT1). The MALAT1, human HCT116, Hela, and HEK293T cell lines have been reduced by up to 98%. The promoter region of the MALAT1 gene has been successfully edited with DECKO,[51,53,54] which is an accepted method aimed at silencing or amplifying gene expression so that the gene loses or regains its function. RNAi with specific small interfering RNAs (siRNAs) are widely used to silence a desired gene encoded by the coding regions of genomic DNA generally in the cytoplasm.[55] However, siRNAs designed to target ncRNA genes, including lncRNAs and miRNA genes, have been discovered to be inefficient, mainly because many lncRNAs are located in the nucleus.[49] Therefore, it has been difficult to achieve successful knockdown of a desired lncRNA gene. Recently, scientists from independent research groups have applied a modified CRISPR/Cas9 system to target ncRNA genes located in the nucleus in the zebrafish genome, resulting in efficient knockdown of a number of ncRNAs, including miRNAs.[56] miRNAs in human cell lines and lncRNAs in animal models, particularly lncRNAs in rats,[52,53] have provided an understanding of the biological roles of ncRNAs in the pathogenesis of schizophrenia. The key feature of the modified CRISPR/Cas9 system is the use of double gRNA, which creates two breaks at certain sites and allows deletion of a larger fragment.[53]

APPLICATIONS OF CRISPR/CAS9 IN SCHIZOPHRENIA AND OTHER DISEASES

Medications form the mainstay of treatment for schizophrenia, and along with psychosocial interventions, they are widely used to manage schizophrenia. However, schizophrenia drugs often cause serious side effects; therefore, a majority of patients with schizophrenia do not take medications, causing this devastating mental disorder to remain uncontrolled. Because a strong genetic component is involved in the pathogenesis of schizophrenia, multiple changes in the genomic DNA of neurons have been implicated as causal factors, but fortunately, great strides have been recently made both in identifying critical genomic regions and in developing advanced genetic technologies. Genetic mutations in the manipulable miRNA and lncRNA genes in the genomic DNA of neurons have been directly linked to schizophrenia. The CRISPR/Cas9 system has provided a powerful method for not only correcting mutations in inherited genetic diseases, but also gene mutations in disease-related genomic DNA, including ncRNA genes. A research team from Duke University recently investigated the application of CRISPR/Cas9 to treat Duchenne muscular dystrophy (DMD), a debilitating genetic disease caused by mutation in one of the exons of the dystrophin gene.[57] Researchers have successfully treated a human disease in a living mouse model for the first time[57] with CRISPR/Cas9 gene editing technology. Similar results from two other research groups from Harvard University and the University of Texas were also impressive.[58,59] These three independent research groups have demonstrated that after restoring one of the exons in the dystrophin gene with the CRISPR/Cas9 technique, correction of the gene can result in restoration of functional dystrophy and increased muscle strength.[56-58] In addition to DMD, the CRISPR/Cas9 method has also been used in various other genetic diseases such as sickle cell anemia and Alzheimer's disease, thereby correcting point mutations, restoring defective genomic DNA, and thus treating the diseases.[60-62] Recently, a research team from Sun-Yatsen University in Guangzhou, China, investigated for the first time the use of the new CRISPR/Cas9 system to edit the thalassemia-causing gene in human embryos.[63,64] As a result, the target gene retains its restorative form in a number of human embryos. Recently, successful application of human induced pluripotent stem cells (iPSCs) using the CRISPR/Cas9 gene editing method has been reported to establish a disease model[65] and effectively treat a number

of diseases including epidermolysis bullosa,[66] β-thalassemia,[67] a1-antitrypsin deficiency,[68] AIDS,[69] Niemann-Pick Type C,[70] and DMD.[71] Therefore, this new approach has held tremendous promise for gene therapy for many other types of diseases, including schizophrenia.

Conclusion

A powerful genome editing tool such as CRISPR/Cas9 has brought many innovative applications and perspectives for both biological research and treatment of diseases, including the complex schizophrenia disease. Application of this technology in schizophrenia-associated ncRNAs will open a new perspective for schizophrenia research to advance our understanding of the biological function of ncRNAs and facilitate the creation of animal models with specific mutations. Since schizophrenia is a complex disorder involving multiple genetic alterations of ncRNAs, the modified CRISPR/Cas9 approach will allow these ncRNAs to be degraded to investigate whether or not disruption of ncRNA genes causes schizophrenia. Besides silencing ncRNA genes, the CRISPR/Cas9 system can deliver regulatory components to target genes, or activate or upregulate target gene expression. The CRISPR/Cas9 method can also allow activation of genes at the transcriptional level, providing researchers with an understanding of the biological role of a specific gene in the development and progression of schizophrenia. Knockout or activation of an ncRNA gene using the modified CRISPR/Cas9 system has several advantages: it is more effective than RNAi and can target multiple genes simultaneously. In regards to schizophrenia-associated ncRNAs miR-137 and Gomafu, it is possible to simultaneously target these two ncRNAs and explore whether miR-137 and Gomafu each, alone, or in combination, influence the development and progression of schizophrenia. The modified CRISPR/Cas9 system opens the possibility of ncRNAs manipulating any exon fragment and exploring the biological function of these ncRNA genes in schizophrenia.

The main bottleneck of schizophrenia research is the absence of animal models to prove the causal relationship between the genetic defects and pathophysiology of schizophrenia, as painstaking work is required not only to model the pronounced symptoms, but also in creating animal models with specific genomic DNA mutations. Due to a number of advantages, the CRISPR/Cas9 system provides a new perspective on developing animal models for schizophrenia research. With the help of the CRISPR/Cas9 system, specific mutations of the target ncRNA gene can be introduced into the embryo and the restorative form of the ncRNA gene can be reintroduced into the gene in a rat or mouse embryo. The rat or mouse and their offspring will contain the mutation or restoration of the original form, allowing researchers to directly compare the symptoms of the resulting experimental and control groups. Disruption of these genes will determine how the development and progression of schizophrenia is affected, thereby identifying underlying molecular pathways in animal models. The created animal models can also be used to test the efficacy of drugs or other potential therapeutic approaches in the treatment of schizophrenia. In fact, it usually takes up to two years for the animal model created by conventional procedures to acquire certain mutations in the offspring because multiple breeding steps are needed, while generating the animal model using the CRISPR/Cas9 approach is less costly and takes about two months. Moreover, in combination with template DNAs and the use of multiple gRNAs, the CRISPR/Cas9 system can introduce any number of mutations into the embryo of an animal or its offspring. With these promising results in mammalian and human cells, CRISPR/Cas9 holds great therapeutic potential for treating human inherited diseases, including schizophrenia and perhaps other inherited mental disorders.

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