EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 7
Issue: 6
314-320
Year: 2020

APPLICATIONS OF CRISPR TECHNOLOGY IN DENTISTRY: A REVIEW

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Article Received on 15/04/2020 **Article Revised on 05/05/2020** **Article Accepted on 26/05/2020**

ABSTRACT
CRISPR, this acronym stands for Clustered Regularly InterSpaced Palindromic Repeats. It is a programmable protein that can edit, eliminate, turn on/off the genome.[1] This modern technology has many potential uses and is standing on the brink of modern medicine which could do a lot in store of future of oral health. Among various versatile genome-editing technologies which can bring sequence-specific modifications into the genomes, most commonly used genome editing technologies are, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and homing endonucleases or meganucleases.[2] CRISPR-Cas9 genome editing technique is still in the stage of infancy which has recently emerged as a potentially powerful tool in the armaments of cancer therapy because of its high efficiency and accuracy[3] By rapidly introducing genetic modifications in cell lines, organs and animals, CRISPR-Cas9 system extends the gene editing into whole genome screening, both in loss-of-function and gain-of-function manners[4] In this review, we discuss an overview of CRISPR-Cas9 genome editing technique and its applications specially in dentistry.

KEYWORDS: CRISPR; Cas9; dentistry; oral cancer**;** immunotherapeutic applications; genome editing.

INTRODUCTION
All cancers arise as a result of changes that have occurred in the DNA sequence of the genomes of cancer cells.[5] Among all, oral cancers have been one of the leading causes of deaths, particularly in the developing countries. The most common form of oral cancer is oral squamous cell carcinoma (OSCC).[6,7] Main reason for this high mortality and morbidity being delay in diagnosis and promt treatment. Recent advances in surgical techniques and diagnostic accuracy have improved the survival of patients with OSCC. However, failure of treatment for OSCC is potentially lethal owing to local recurrence, regional lymph node metastasis, and distant metastasis.[8,9] Therefore, there is requirement of advancing technology to treat cancer patients from the root cause. CRISPR is the first-ever treatment that genetically alters a patient's own cells to fight cancer. Moreover, CRISPR technology has several potential uses in dentistry other than treatment of oral cancer. Although its practical applications in clinical setting may be a long time coming, this technology could bring permanent changes in dental field. As the name suggests Clustered Regularly Interspaced Short Palindromic Repeats, **clustered** - a group of several discrete items that are close to each other, **regularly** – having a constant pattern, **interspaced** - interval between two things, **short** – small distance from one end, **palindromic** – a stretch of DNA in which the sequence of nucleotides on one strand are in the reverse order to that of complementary strand, **repeats** – recur. Genetic modifications are introduced in present diseased DNA sequence and CRISPR-Cas9 extends the gene editing into whole genome screening. CRISPR-Cas9 can accurately edit genes, not only in cell culture models and testing animals but also in humans, this ablity allows its use in therapeutic traverse. Moreover, CRISPR-Cas9 can be employed to rapidly engineer immune cells and oncolytic viruses for cancer immunotherapeutic applications.[3]

History
CRISPR repeats were first discovered by accident in 1987 by Osaka University researcher **Yoshizumi Ishino** and his colleagues, in the Escherichia coli genome during an analysis of genes involved in phosphate metabolism.[10.11] Similar repetitive sequences were identified in other E.coli strain and the closely related enterobacteria, including Shigella dysenteriae and Salmonella enteric.[11] Time line of some important key studies in discovery of CRISPR-Cas9 system is given in Table 1.

Year	Discoveries in CRISPR- Cas9 Systems
1987	First discovery of CRISPR (Ishino et al.)[12]
2000	CRISPR was found widespread in both bacteria and archaea (Mojica et al.)[13]
2002	Proposed with a name CRISPR (Jansen et al.)[14] Discovery of CRISPR transcripts (Tang etal.)[15]
2007	First experimental evidence for CRISPR adaptive immunity. (Barrangou et al.) [16]
2013	Demonstration of Cas9 genome engineering for the first time in eukaryotic cell. (Cong et al.)[17] (Mali et al.)[18]
2018	A study was carried out (Huang et al.)[19] showing potential ability of CRISPR- Cas9 system in reduction of pro-tumorigenic behaviour in human OSCC.

Since its discovery, the CRISPR-Cas9 technique has been widely and successfully applied in many areas of biomedical research, such as gene activation and repression, improved animal model construction, functional screening, and novel therapeutic development.[13]

Mechanism Of Crispr Genome Editing

Clustered regularly interspaced short palindromic repeats-Cas9 technology found in bacteria and archaea is a RNA guided genome editing tool, has its dawning from immune defence mechanism, which provides immunity to the host against invading nucleic acids such as viruses and phages.[16] According to the most popular classification, there are three types of CRISPR/Cas systems.[20] Type II CRISPR/Cas system is most commonly used and consists of three components: an endonuclease (Cas9), a CRISPR RNA (crRNA), and a transactivating crRNA (tracrRNA). The crRNA and tracrRNA molecules together form a duplex structure called the guide RNA (gRNA). Guide RNA can be replaced by a synthetic fused chimeric single gRNA (sgRNA), which simplifies the use of CRISPR/Cas9 in genome engineering.[21]

The sgRNA contains a unique 20 base-pair sequence followed by a short DNA sequence termed the "protospacer-adjacent motif" (PAM) that is designed to be harmanious to the target DNA site.[3,4] PAM is a special marker for Cas9 to distinguish the target DNA from its own genome, lack of PAM will lead to failure of unwinding the target.[4] sgRNA and Cas9 nuclease form a ribonucleoprotein (RNP) complex when expressed in the cell, guided by the sgRNA to a target DNA site. Cas9 precisely cleaves the DNA to produce a double stand break (DSB), generating blunt ends and sgRNA binds to the target sequence by Watson-Crick base-pairing.

DSBs in eukaryotes are repaired by one of two endogenous repair mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR) depending on the cell state and the presence of a repair template. The HDR pathway results in accurate repair by using a donor DNA template that is recombined at the DSB site. In the absence of a homologous repair template NHEJ usually result in (insertions or deletions) of random base pairs disrupting the target sequence.[22]

Once DSBs are formed further gene editing is done depending on the diseases we aim to treat. Gene correction, inactivation, ablation of duplicated genes, insertion or T cell engineering is done and these cells are transplanted back into the same patient by various delivery systems.

Thus, CRISPR/Cas9 technology allows precise and highly structured cleavage of a desired target DNA sequence giving the relative ease and simplicity of designing sgRNAs. An added advantage of this technology is its potential for multiplexibility through the use of different sgRNAs.[23]

Applications of Crispr-Cas9 Genome Editing

CRISPR/Cas9 system has attracted considerable attention because of its various advantages in genome editing, and scientists gradually consider it to be a powerful therapeutic tool for treating diseases associated with genome mutations.

Cancer

Cancer is a complex disease that derives from a variety of genetic and epigenetic changes, and is a major threat to human life and public health.[24] CRISPR/Cas9 with its ease of use can be used to treat cancer by increasing the production of therapeutic immune cells, such as construction of chimeric antigen receptor T (CAR-T) cells and programmed cell death protein 1 knockout.[25] The generation of CAR-T cells is one of the most striking applications of CRISPR-Cas9 technology in cancer immunotherapy. In general, CAR contains an intracellular chimeric signalling domain capable of activating T cells and an extracellular single-chain variable fragment that can specifically recognise tumour antigens.[26, 27] These genetically modified T cells carrying tumour-targeting receptors have achieved positive therapeutic outcomes in patients with various haematological malignancies such as leukaemia and lymphomas.[28-30]

The PD-1 protein and programmed cell death ligands (PD-Ls) are key to negative regulation of the immune system, specifically on T-cells. Their attenuation of the immune response helps tumor cells survive by evading the immune system and significantly increase the overall survival rate in cancer patients.[31]

Cardiovascular Diseases

Treatment of CVD in practice is more concerned about relief of disease symptoms and correction of morphologic defect in the heart without addressing

potential genetic defects. Presently, with the help of CRISPR/Cas9 in-depth analysis of CVD pathogenic genes as well as their molecular mechanisms has made it possible to test the ability of gene therapy to control specific gene expression and improve gene functions. With the help of genome editing technologies, various research models of cardiovascular conditions have been created for the treatment.

Metabolic Diseases

Metabolic diseases refer to the pathological state in which the body's carbohydrates, protein, fat, etc. are metabolically disordered. Metabolic diseases include a group of syndromes that are caused by both genetic factors and the environment.[32] Gene editing technology can be applied in functional gene screening, gene therapy and the construction of metabolic disease models, such as obesity, diabetes, and hyperlipidemia. Leptin (Lep) is a hormone secreted by white fat cells that acts on the metabolic regulation center of the hypothalamus through the leptin receptor (LepR).[33]

Neurodegenerative Diseases

Neurodegenerative diseases (NDs), at least including Huntington's disease (HD), Alzheimer's disease (AD), and Parkinson's disease (PD), are a group of conditions that have attracted the most concern because there have been no specific diagnostic approaches or established treatments for them.[34,35] Clinical features of NDs are a result of (potential pathogenic mechanisms) complicated interactions of multiple genetic factors; either alone or in combination. The emergence of gene editing platforms provides a convenient approach to study gene functions related to NDs.[36]

Viral Diseases

Recently, CRISPR technology has emerged as antiviral therapeutics for treating infectious diseases, either by altering the host genes required by the virus or by targeting the viral genes necessary for replication. Genome editing-based HIV therapy has involved modifying infection-related genes to produce HIV-resistant CD4+T cells and subsequently reinfusing the edited cells into patients[37] Human papillomavirus (HPV) oncogenes E6 and E7 expression is implicated in malignant transformation and is strongly associated with cervical cancer. Therefore, targeted mutagenesis of those high-risk HPV genes by gene editing tools may be a potential genetic therapy and may reverse cervical cancer in situ.[37]

Haematological Diseases

CRISPR–Cas9 genome-editing tool can alter the DNA of bone-marrow stem cells, offering a potential treatment for certain blood diseases. As a consequence of indels introduced by NHEJ-mediated DSB repair, the resulting frame shift or nonsense mutations can give rise to truncated proteins that have a gain- or loss-of-function.[38] CRISPR/Cas9 gene editing has been used to modify a subset of blood stem cells to reverse the clinical symptoms of sickle cell disease and beta-thalassemia.[39] Treatment of leukemia, lymphomas, multiple myeloma is in current clinical trials in hematology using the CRISPR/Cas9 system.[40]

Other Hereditary Diseases

As a result of engineered nuclease mediated editing of genomic modifications, other animal disease models have been developed, simulating hereditary eye disease, duchenne muscular dystrophy, Rett syndrome, hereditary deafness, Wilson disease, Laron syndrome, Niemann–Pick disease, Netherton syndrome, and so on. Further advancement in applications of genome editing technologies will take place covering animal models in disease mechanism research and treatment development.[37]

Applications of Crispr/Cas9 Technology In Dentistry

CRISPR can diagnose and treat various oral pathologies by identification of causative organisms or faulty genes and manipulation of gens. CRISPR will also help to identify genes that suppress the tumor-promoting properties of the genes that cause oral cancer. Various applications of CRISPR in dentistry till date are as follows:

Dental Caries

Streptococcus mutans is the keystone aetiological agent of human dental caries.[41] Normal bacterial flora is already present in the oral cavity, but when this oral flora exceeds in number there is initiation of disease. Therefore, we should understand how to modulate bacterial composition that achieves a healthy, dynamic balance of the oral ecosystem. Dysbiosis of the biofilm, with changes in the bacterial composition and especially accumulation of S. mutans, results in demineralization of the tooth surface and the eventual occurrence of dental caries.[42] Although different antimicrobial strategies, such as antibiotics, antimicrobial peptides (C16G2 that specifically targets S. mutans in the oral microbiome) 37 and lytic bacteriophages, could offer partial solutions, however precise and programmable strategy that can distinguish among closely related microorganisms, and that allows for fine control over the composition of a microbial population is still to be acquired[43] Researchers have used RNA-guided nucleases (RGNs) CRISPR/Cas technology to produce antimicrobials, whose range of activity is chosen by design. RGNs also enables modulation of complex bacterial populations by selective knockdown of targeted strains based on genetic signatures.[44]

Dental Plaque

Role of Streptococccus Mutans[43] Major virulence factor of streptococcus mutans, glucosyltransferases (Gtfs), utilize sucrose to synthesize extracellular polysaccharides (EPS), which leads to the formation of dental plaque biofilm[43]. Formation of biofilm is the first step in progression of dental plaque formation. In a study conducted by Gong T et al, self-targeting CRISPR arrays

were designed (containing spacer sequences identifying with gtfB) and cloned onto plasmids. This plasmids were transformed into UA159 (self-targeting) to acquire desired mutants. This resulted in high reduction of EPS synthesis and eventually breakdown of biofilm formation.

Role of Porphyromonas gingivalis[45] Porphyromonas gingivalis a Gram-negative anaerobic rod has been identified as a foremost pathogen causing microbial dysbiosis. Almost 95% of clinical strains of P. gingivalis has been found to bear CRISPR arrays. It is possible that this genetic immune system of bacteria participates in modulating the microbiome of 'chronic' periodontitis, and may protect itself in the periodontal pocket where bacteriophages are abundant and even out-number bacteria. Therefore, CRISPR technology can be of great use and help as a promising tool for dental clinics in order to prevent dental plaque formation and eventually prevent periodontitis.

Oral Cancer

Oral squamous cell carcinoma (OSCC) which is the most common type of oral cancer has remained a disease with poor survival for decades with few exceptional treatment options. CRISPR is an emerging technology that genetically alters patient's own cells to fight cancer. Researchers are more focused on few genes that mutate at very high rate, but there is another class of slower mutating gene that can also lead to tumors. A team of researchers in Canada have identified 15 tumor suppressor genes that can trigger rapid growth of human head and neck squamous cell carcinoma (HNSCC) when they mutate.[46]

Kiyosue et al. 2013, in his study examined p75 neurotrophin receptor (p75NTR) expression immunohistochemically in oral leukoplakia (OL), the most frequent precancerous lesion, and OSCC. Result of this study suggested that p75NTR is expressed in undifferentiated cell populations in OL and OSCC. This study also concluded that p75NTR is possibly involved in invasion and poor prognosis in OSCC.[8] Ludwig M et al. 2017, introduced Genome-scale CRISPR-Cas9 Knock-Out (GeCKO) libraries into OSCC cell lines and six/14 (43%) of the cell lines were responsive to the combination.[47] Huang et al. 2017, investigated the importance of the p75NTR in human tongue squamous carcinoma cells by using CRISPR/Cas9 technology. According to this study, several tumor promoting properties of SCC-9 cells are suppressed by deletion of p75NTR, suggesting that p75NTR is a potential target for the growth of novel treatment modalities for tongue cancer.[9] CRISPR/ Cas9 system is having great efficacy in identifying genes associated with oral cancer pathobiology and in treatment of the same by gene knockout technique.

Salivary Dysfunction

Previous study has demonstrated the role of water-specific protein aquaporin 1 (AQP1) gene expression has chief role in correction of salivary dysfunction in cancer patients receiving ionizing radiation treatment.[48] Wang Z et al., used CRISPR/Cas9 system to enhance AQP1 gene expression by designing gRNA sequence and cytomegalovirus (CMV) (endogenous promoter) containing homology directed repair (HDR) template.[49] He suggested that salivary gland dysfunction can be potentially treated by replacing endogenous promoter.[49]

Recently, CRISPR/Cas9 system has been successfully used to target important genes in many cell lines and organisms and may be possible to develop successful mesenchymal stem cells -derived therapy for primary Sjogren's syndrome.[50]

Tooth And Palate Development

Use of CRISPR/Cas9 system has been used to reveal importance of C-terminal domain of Msx1 gene in tooth and palate development. Mitsui S. N. et al, targeted Msx1 in mice with CRISPR/Cas system, homozygous mice exhibited agenesis of lower incisors with or without cleft palate [51]. MSX1 homeodomain also has been identified in non-syndromic tooth agenesis predominantly affecting premolars and third molars.[52]

Herpes Virus

Almost 100% of the adult human population carries herpes viruses (large DNA viruses). Herpesviruses include several important human pathogens causing oral lesions, such as herpes simplex viruses (HSV) type 1 and 2 (gingivostomatitis, herpes labialis, mucocutaneous ulcers) human cytomegalovirus (HCMV) (infectious mononucleosis), and Epstein-Barr virus (EBV) (hairy leukoplakia, mucocutaneous ulcers). Though antiviral drugs are used to treat these infections, complete clearance of virus has not achieved.

Recently, CRISPR/Cas9 system has been used to target and alter specific regions within the genome of virus-infected cells. Inactivation, inhibition of viral replication or in some cases eradication of viral genome from infected cells has been achieved.[53]

Temporomandibular Disorders And Overlapping Pain Conditions

CRISPR/Cas9 system can treat temporomandibular disorders by targeting genome modification. Seventh Scientific Meeting of The TMJ Association, 2014, in Bethesda, MD presented their latest findings regarding TMD and its accompanying overlapping pain conditions research. Presenters discussed the use of exciting new technologies including CRISPR/ Cas9 methods to engineer RNA guided transcriptional activators and repressors targeted to human genes that are already linked to pain pathways.[54]

In Chronic Pain

Chronic pain in various orofacial diseases is the concern of many dentists. Various medications from NSAIDs to opioid provide symptomatic relief for some time and pain is again aggravated after effect of medication is gone. Certain mutations in this gene were found to deprive the ability to feel pain in the affected individual. The reason for this, mutated gene downregulate the passage of the pain signals across the neural pathway, by control of certain molecules involved in this process, found on the cell surface of the neurons.[55] With the help of CRISPR epigenetic marker that activated this pathway can be edited.

In Paediatric Dentistry

Oro-facial abnormalities coming from genetic etiology is beyond real help for dentists as we cannot modify a genetic defect but workout a treatment plan that alleviated the problem. With CRISPR we can manipulate the genetic structure to produce a normal genome sequence. In near future by identifying the faulty genes for a specific disorder, we may find a CRISPR solution to the fault can revise the genetic structure of the genome.

Limitations and Disadvantages Of Crispr Technology

1. Despite of advancement that has been made in CRISPR-Cas9 technology, safety and efficiency are important concerns that still require comprehensive studies.
2. Developing a CRISPR-Cas9 protocol can be challenging and time consuming.
3. Target site selection and sgRNA design are not as simple.
4. gRNAs tend to have relatively high mismatch tolerance and therefore Cas9 commonly cleaves sequences similar to those of target genes (off-target sites).[56]
5. Patient to patient variability - Cas9 antibodies are naturally present in the serum of some donors, which can cause low editing efficiency and could lead to a serious immune storm in patients receiving CRISPR-Cas9 treatment.
6. Variations in genes of different patients may also lead to unexpected off-target gene editing even with well-designed gRNA.
7. Generalizability of CRISPR-Cas9 technology is not excellent.[57]
8. Unexpected and unintended results may come in highly experimental treatment involving severely ill patients.[58]
9. ‘Genetic drive’ biggest fear of CRISPR – after manipulation or incorporation of the genes, these genome sits within cells which potentially can be transferred on to other organisms and passed on generation to generation.
10. Though CRISPR can treat various diseases and also has its limitations, it cannot be a treatment of choice for poor people as it is very expensive.

CONCLUSION

CRISPR/Cas9 system is a precise technique to treat various diseases including oral cancer, caused by abnormality in genes. It is a DNA free approach suited in both in vivo and in vitro. Though it requires optimization (efficacy, safety, & specificity) this technology treats diseases from its root cause that is treating genes in various ways. In dentistry, it has role in identification of causative organisms or faulty gene in various oral pathologies mentioned in this article. CRISPR can treat these oral diseases either by modulation or gene knockout of (causative) complex bacterial populations or faulty genes. Although treatment of oral diseases has started at research level, the practical application of those uses in the clinical practise is still in the stage of infancy which could stand to change dentistry permanently. Present pandemic COVID 19 which has spread all over the planet, CRISPR based test for its diagnosis has been approved from FDA recently. CRISPR can quickly find and link onto any genetic sequence in a sample, gives results within an hour. In this way, from diagnosis to treatment CRISPR can achieve astonishing goals for mankind.

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