Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and Its Current Applicatıons in Microbial Diagnosis

Düzenli Aralıklarla Bölünmüş Palindromik Tekrar Kümelerinin (CRISPR) Güncel Mikrobiyal Tanıda Kullanımı

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Özet

Nanoteknolojiler, bilişim teknolojileri, genetik teknoloji, sentetik biyoloji, rejeneratif tıp, robotik uygulamalar, nöroteknoloji ve yapay zeka son yıllarda hızlı gelişen ve tıbbi uygulamalara yenilikler getiren teknolojilerden bazılarıdır. Bunlar sayesinde pek çok sağlık sorununa daha kolay tanı koymak ve daha iyi tedavi etmek mümkün hale gelmiştir. Yeni tıp teknolojilerinin asıl amacı hastalıkların tanı ve tedavisini sağlamak olsa da aynı teknolojiler sayesinde daha güçlü, daha sağlıklı, daha uzun yaşama olasılığı olan insan haline gelme olanağı da sağlanmış olmaktadır. Bu durum gelişen teknolojilerin hızla kullanıma girmelerine, amacına uygun olmayan şekilde tüketilmelerine ve pek çok etik soruna yol açabilmektedir. Ortaya çıkabilen etik sorunlar üç ana başlık altında toplanmaktadır: Hasta güvenliği sorunları, kaynakların adil kullanımı sorunları ve norm değişikliği sorunları. Yeni teknolojilerin özellikle ilk kullanım dönemlerinde kullanıcıların beceri eksikliğine bağlı olarak ölümlere kadar gidebilen önemli komplikasyonlar görülebilmektedir. Yeni teknolojilerin kullanımı konusunda toplumun varlıklı ve güçlü kesimleri daha ayrıcalıklı olduğundan hakkaniyetli bir paylaşım söz konusu olmamakta, bu da yeni insani sorunlara yol açabilmektedir. Bazı yeni teknolojiler ise hastalık tedavisi dışında daha güzel ve daha genç görünmek, daha keskin bir hafızaya sahip olmak, fit olmak gibi tıp dışı nedenlerle kullanılabildiğinden bireysel normları değiştirmekte ve hizmet sunanlar üzerinde bu doğrultuda bir toplumsa baskıya neden olmaktadır. Öte yandan bu teknolojilerin gelişmesinin ve yaygın kullanımının iyi ve gerekli olduğunu savunan görüşler de bulunmaktadır. Söz konusu etik sorunların hiç birisi teknolojinin kendisi ile ilgili olmayıp, onu kullananların bilgi, tutum ve becerilerinden kaynaklanmaktadır. Bu durum tıp eğitiminde ve uygulamalarında etik konulara daha fazla yer verilmesinin önemini göstermektedir.

Anahtar Kelimeler: Tıp teknolojisi, Etik sorunlar, Hasta güvenliği, Hakkaniyet

Abstract

Nanotechnologies, information technologies, genetic technology, synthetic biology, regenerative medicine, robotic applications, neurotechnology and artificial intelligence are some of the technologies that are developing rapidly in recent years and bring innovations to medical applications. New medical technologies enable us better diagnose and better treat many health problems. Although the main purpose of new medical technologies is to provide diagnosis and treatment of diseases, the same technologies enable us to become stronger, to live healthier and longer. This situation can lead to rapid and frequent use of developing technologies beyond purpose and may cause many ethical problems. Ethical problems that may arise are grouped under three main headings: Patient safety issues, unfair use of resources and problems of norm change. Significant complications can be seen, especially in the initial use periods of new technologies due to lack of skills of users. As the wealthy and powerful segments of the society are more privileged in the use of new technologies, there is no equitable sharing, which can lead to new humanitarian problems. Some new technologies change the individual norms and cause social pressure on the service providers, because they can be used for non-medical reasons such as looking more beautiful and younger, having a sharper memory and being fit. However, none of these ethical problems are related to the technology itself, but the knowledge, attitude and skills of those who use it. This demonstrates the importance of more ethical issues in medical education and practice.

Keywords: Medical techologies, Ethical issues, Patient safety, Equity

Introduction

To date, conventional methods for microbiological diagnosis were culture, serology, biochemical, MALDI-TOF and PCR based methods. Today they are well known and their routine application is being done seamlessly. However, in recent years new diagnostic methods that involve gene editing are being developed. Gene editing is to change the genome of an organism in various ways like inserting, deleting or changing the genomic nucleic acid sequence. A group of methods are used to achieve this goal which one of them is the newly discovered CRISPR-Cas method. CRISPR-Cas systems include several methods which DNA sequences of the subject organism is targeted. After discovery in prokaryotes as a defense mechanism, CRISPR-Cas based systems attract attention in recent years also as a potential diagnostic tool for human diseases. Here in this review, we will introduce current state of CRISPR-Cas systems and provide insight into the application of them in microbiological diagnostics.

Prokaryotic cells have several immune defense systems named innate immunity, adaptive immunity and cell suicide/dormancy. Innate immunity was the first discovered one which utilizes well known restriction endonucleases that cleaves foreign nucleic acids without enhanced specificity and memory. With adaptive immune system, enhanced specificity and memory features of prokaryotic immune system becomes available. Suicide and/or dormancy based immune defense systems utilize cell death and dormancy of infected prokaryotes which are triggered by infection if innate and adaptive immune systems fail. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) system is the latest discovered feature of prokaryotic adaptive immune system. Briefly in CRISPR-Cas system, first step is integration of foreign DNA to CRISPR-Cas loci, followed by transcription of this DNA to micro RNA for guiding the specific endonucleases (Cas enzymes) to target and bind to foreign nucleic acids, and finally cleavage of the targeted nucleic acid by Cas enzymes[1].

Discovery of CRISPR-Cas system

CRISPR-Cas systems are sequence specific and RNA guided endonuclease systems which can cut the targeted nucleic acids at a specific point. These systems are discovered in bacteria as an adaptive immune and defense system against foreign nucleic acids especially attackers like bacteriophages. In 1987 unusual repeat sequences was found to form the central role for CRISPR-Cas system separated by non-repeating sequences. They were firstly described in Escherichia coli (E. coli)[2]. Repetitive DNA sequences were also observed in archaea, particularly in Haloferax mediterranei in 1993[3]. Intervening non-repeating (also called spacer) sequences between repetitive CRISPR sequences which thought that originated from previous infection agents and suggesting and adaptive immunity feature were discovered in 2005[4]. Although precise functions of these repeat and intervening sequences as well as related Cas genes which are endonucleases are understood in 2007[5]. In the same year repetitive DNA sequences of Mycobacterium tuberculosis strains were used as genetic markers for utilizing them in bacterial strain differentiation[6]. To date, in almost half of the bacteria and 90% of the archaea CRISPR-Cas systems are identified[5]. And two parts of the system is identified. CRISPR part of this system functions as an immunologic memory and the second part is the Cas proteins (endonuclease enzymes) which cleave foreign nucleic acid of future attacks by using the guidance of the immunologic memory.

CRISPR-Cas system classification

Although CRISPR-Cas systems may be classified in different bases, Savitskaya et al.[7] have published a classification based on protein composition of CRISPR-Cas systems. Latest information divides CRISPR-Cas systems into two classes (Class 1 and Class 2), five types and 16 subtypes[8]. Class 1 CRISPR-Cas systems contain multi-subunit proteins while Class 2 contains one protein.

Class 1-type I systems includes Cas3 as signature protein. Cas3 acts as both endonuclease and helicase. Endonuclease

activity cuts nucleic acids while helicase activity unwinds DNA-DNA and DNA-RNA duplexes. Type I systems also have multi-subunit crRNA which enables them to recognize multiple targets. Class 1-type III systems are typically including Cas10 as signature protein. Cas10 also has several domains like cyclases (synthesizes cAMP which is a key second messenger) and polymerases. An important feature of type II systems is they can cleave both DNA and RNA. Class 1-type IV is characterized by including csf1 gene. Mechanism of type IV systems require better understanding since current literature is not sufficient.

Class 2-type II systems includes Cas9. Like type I systems, type II systems also require protospacer adjacent motif (PAM) for target recognition. Class 2-type V typically include Cpf1 protein which is similar to Cas9. Type V systems also require PAM for target recognition[9–11].

Cas1, Cas2, Cas3 and Cas4 are first identified and the most conserved of Cas proteins. As endonucleases, Cas proteins are highly differentiated as a result of high adaptation speed of defense systems of bacteria. Analyses have shown that approximately 65 different sets of Cas proteins present[12,13]. Another discovered protein group related to CRISPR-Cas systems are sequences named Repeat Associated Mysterious Proteins (RAMP). They were identified as containing RNA recognition domains but not identified as Cas proteins[13]. All CRISPR-Cas system types work with crRNA that leads Cas protein to locate and bind to targeted foreign nucleic acid sequence. Due to high degree of diversity it is a hard task to classify all known and newly identified Cas proteins. Discrepancies in the classification of CRISPR-Cas systems and cas genes urged researchers to construct a standard classification and nomenclature14.

Action Mechanism of CRISPR-Cas system

CRISPR-Cas DNA in prokaryotic sequence has two main parts: identical palindromic repeats and unique spacer (originated from invaders) sequences[4]. The prokaryotic cell acquires spacer sequences from invader (mostly viral) sequences after cutting and integrating them to its own genome, if survives from invasion[15]. These sequences provide adaptive immunity to prokaryotic cell for defense against future attacks. When attacked with same virus later, matching of newly attacking viral sequences with spacer sequences from earlier attacks triggers Cas proteins that starts the action of CRISPR-Cas system for cleaving the newly attacking viral genome. In this stage protospacer adjacent motif sequences (PAM) distinguish prokaryote's own genomic sequence from invader's sequence for not to attack its own genome. PAM consists of 2–5 base pairs and can be located either in 5' end or 3' end of spacers[16].

Cas genes are also important components of bacterial immunity based on CRISPR-Cas system. Cas protein group is a huge and varied enzyme group to interact with and cut foreign nucleic acids. And also Cas proteins have a feature of RNA-directed activity which enables them to target foreign nucleic acid with the guidance of RNA sequences (crRNA)[17]. A Cas enzyme acts as a pair of 'molecular scissors' that can cut foreign nucleic acids at a specific location. crRNA helps Cas enzyme to recognize a specific sequence of foreign nucleic acid to cut.

Stages of CRISPR-Cas system action are acquisition, expression and interference. In the acquisition stage, which is not a totally explored stage yet, nucleic acid of foreign invader is integrated into the CRISPR-Cas locus of the host genome as new spacers. After integration of new spacer sequences, also new repeat sequences are introduced to both 3' and 5' ends of the spacer sequences. In this acquisition step of new foreign nucleic acid Cas1 and Cas2 take place. In the expression stage, CRISPR-Cas locus is transcribed to a specific crRNA for recognition of targeted nucleic acid and directing Cas enzyme to it. Finally in the interference stage, Cas protein complexes (enzymes) which accompanied by crRNAs interfere with and degrade the targeted foreign genome[18].

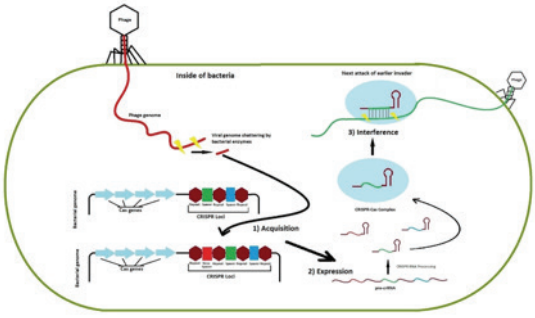

Figure 1 Mechanism of CRISPR-Cas stages

Prospective applications of CRISPR-Cas system

Identification of pathogenic agent is of crucial importance for microbial diagnosis and treatment of infectious diseases. Currently microbiological diagnosis methods may be classified as direct methods, cultured methods, serologic methods, MALDI-TOF MS and genetic based methods. Alternatively, these tests can be grouped as; (1) clinical laboratory tests which are relatively complex and require specialized facility, equipment and personnel, (2) relatively simple tests which don't need specialized facility and equipment and (3) simple tests which patients can do themselves. Microscopy is the most common direct technique for diagnosis that may detect and identify the pathogen. Culture is a specialized media for isolation of infectious agents. There are two different types of culture method; selective and non-selective. Selective media contain inhibitory materials that permit only the growth of specific types of microorganisms. Nonselective (non-inhibitory) media permit the growth of many microorganisms. Hepatitis virus, Epstein-Barr virus, human immunodeficiency virus type 1 (HIV-1) infections etc. can be detected only serologically, because they cannot be grown any known culture technique. There are some sub-methods of serologic diagnosis such as agglutination, complement fixation and ELISA (enzyme-linked immunosorbent assay).

Recently researches are much focused on preventing the spread of acute pandemic viral infections like Zika Virus (ZIKV), Ebola Virus (EBOV), Dengue Virus (DENV), etc. With the help of CRISPR-Cas methods discovery of new diagnostic methods and drugs are aimed to prevent mentioned infectious diseases. Also, laboratory experimental work of the biotechnological research is also can be accelerated and simplified by the utilization of CRISPR-Cas methods. Development of novel microbiological diagnosis systems like CRISPR-Cas based ones are belong to area of synthetic biology research and development.

The most well-known and extensively used CRISPR-Cas system is CRISPR-Cas9. In 2013 Sampson et al.[19] demonstrated that Cas9 of Francisella novicida (FnCas9) is able to target the mRNA of bacterial lipoprotein (BLP). In host (eukaryotic) innate defense system BLP triggers the inflammatory cascades of the processes which starts with Toll-like receptor 2 (TLR2). Although when Cas9 blocks mRNA of BLP, TLR2 can't start the immune inflammation process thus permits the virulence of F. novicida[19,20]. This study highlights the possible use of CRISPR-Cas system as a post-transcriptional modification system. Also, RNA targeting feature of Cas9 may be used to target viruses which contain RNA.

In eukaryotic RNA interference (RNAi) machinery small RNAs disable mRNA of certain genes to regulate gene expression. Also, RNAi inhibits RNA of the eukaryotic viruses as a eukaryotic defense system. Although some viruses have developed ways to overcome eukaryotic RNAi, Cas9 of CRISPR-Cas system has the potential to inhibit RNA of the eukaryotic viruses apart from RNAi. In another study in 2015 Price et al.[21] showed that FnCas9 together with RNA-targeting guide RNA (rgRNA) is able to inhibit the expression of viral protein by 50-60% in hepatoma cell lines which infected with HCV. In this example inhibition of HCV viral RNA by Cas9 is done by engineered CRISPR-Cas9 machinery. Similarly, inhibition of human immunodeficiency virus (HIV), hepatitis B virus (HBV), human papillomavirus (HPV), Epstein-Barr virus (EBV) and geminiviruses are under research for combat with

them by the usage of engineered CRISPR-Cas system. Taken together, these studies show the promising future applications of CRISPR-Cas systems as an antiviral tool.

Although before using Cas9 as an antiviral human therapeutic agent, how to deliver the Cas9 to human cells must be known. Price et al.[5] suggest that this issue may be solvable by developing delivery systems which allow transient expression of CRISPR-Cas9 system (especially guide RNA of this system which guides Cas9 to targeted nucleic acids). The other issue which is needed to be overcome is the need for an effective delivery system as expression of Cas9 and related guide RNA must only happen in the targeted tissue and cells. Viral vectors and non-viral delivery systems are in the stage of research currently to overcome these two issues.

CRISPR-Cas system has alternative activities resulting from several different Cas proteins that has different properties as endonucleases. As examples, Cas9 which is most widely used and known endonuclease uses a guided RNA to cut foreign DNA. Cas9 is also most used CRISPR system in laboratory research tool for genome editing[22]. Cas12a (also known as Cpf1) can be programmed to target and cut foreign double stranded DNA (dsDNA) while Cas13a (also known as C2c2) can be programmed to target and cut RNA. Catalytic activity of Cas12a and Cas13a differs from Cas9 by having unspecific cleavage activity that can cut RNA sequences in either 5' and/or 3' direction of the targeted nucleic acid upon recognition of the target sequence[23]. This feature is called collateral cleavage. In 2016, East-Seletsky et al.[24] showed that Cas13a is able to cut a reporter RNA that has a fluorophore and a quencher molecule which gives fluorescent signal after cutting, showing that CRISPR maybe used in conjunction with Real-Time PCR. Thus, currently it is proved that CRISPR-Cas adapted Real-Time PCR can be used for diagnosing pathogens by using their genome.

However, pathogen genome detection diagnostic tools are needed to be very sensitive since sometimes nucleic acids in the specimens taken from patients exist in very small amounts. To overcome this issue and enhance the sensitivity of detection, Gootenberg et al.[25] developed SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing) method. For detection of viral and bacterial nucleic acids in attomolar (10-18 mol/L) level they combined Cas13a activity with isothermal amplification of the targeted nucleic acid. Current PCR based diagnostic systems can detect in femtomolar (10-15 mol/L) level. Isothermal amplification is the amplification of nucleic acids in a constant and low temperature that is possible with a cost-effective and portable diagnostic tool without standard PCR equipment. Maybe one of the most remarkable feature of SHERLOCK, as Gootenberg et al.[25] showed, is the ability of detecting and distinguishing specimens with only one nucleotide difference. A potential limitation of CRISPR-Cas systems that uses Cas13a is the unstable molecular structure and susceptibility of RNA to RNases. This makes the usage of RNA either in laboratory or portable equipment difficult. Also in SHERLOCK, collateral cleavage property of Cas13a is used to cleave the sequence which involves fluorescent reporter that is activating when cleaved, indicating the presence of interested sequence.

On the other hand, in an article written by Chen et al26, authors showed the endonuclease cleavage activity of Cas12a on DNA, eliminating the disadvantage of SHERLOCK which comes from utilizing of RNA as a sensitive nucleic acid to work with in diagnostic methods. In their method they used Cas12a together with isothermal amplification and is named as DETECTR (DNA Endonuclease Targeted CRISPR-CAS Trans Reporter). DETECTR system utilizes RNA-guided DNA cleavage activity by Cas12a with isothermal amplification. It was shown that DETECTR can detect human papilloma virus in patient samples rapidly and specifically with a relatively simple method26. HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases) method was introduced by Myhrvold et al. which is another diagnostic tool for detec-

tion of viral nucleic acids[23]. In this method heat and chemicals inactivate ribonucleases (RNases) and other enzymes which may decrease the detection efficiency of nucleic acids to be diagnosed in the patient specimen's working solution. After application of HUDSON method SHERLOCK procedure takes place and diagnose the nucleic acid of interest on the paper strips. In HUDSON method, paper strip application can be done in the field easily, which is the virtue of this method. With combination of HUDSON and SHERLOCK, as small amounts as 1 copy per microliter of Dengue Virus (DENV) and Zika Virus (ZIKV) nucleic acid in patient samples can be detected directly, faster and easier in the field[27]. In addition, small amount of body fluids such as serum, whole blood, saliva etc. are enough for diagnosis in a duration of < 2 hours with this combined method.

On the other side, CRISPR-Cas systems can be used for gene regulation such as repression or activation of the interested genes which are under investigation in research study laboratories. For example, RuvC and histidine-asparagine-histidine (HNH) are two important endonuclease domains of Cas9 protein that performs cleavage activity of foreign nucleic acids. Mutations at these domains can be used to create inactive Cas9, which can bind to targeted DNA without cleaving. When targeted and bind to interested gene's promoter, inactive Cas9 can act as a repressor for gene expression. This method is called CRISPRi[28]. Inactive (without cleavage activity) Cas9 (dCas9) represses expression by binding to promoter or open reading frame and blocking transcription of the target gene. Some research groups successfully used this dCas9 method for repression of β-galactosidase expression in Streptococcus pneumonia[29] and for identification of basic genes for mycobacteria[30]. dCas9 can also repress more than one gene at the same time by binding multiple promoters.

At the same time, when needed in research study experiments gene transcription can be activated in cells by the transcription activator (transcription factor) VP16 (Virus Protein 16 is a transcription factor encoded by the UL48 gene of Herpes simplex virus-1) or by p65 transcription factor of adenovirus p65AD) together with dCas9[31]. These sort of CRISPR-Cas systems that works with transcription factors also have synergistic activation features which enables them for usage simultaneously on the same target. In addition, CRISPR-Cas systems can also be used as a marker through fusing with green fluorescent protein (GFP) to determine localization or co-localization of molecules that is under investigation[32].

The main advantages of the all CRISPR-Cas based diagnosis tests are reduced test duration, relatively simple protocol and reduced cost as well as improved sensitivity and ability to perform the test in field with simple instruments when compared to conventional IVD.

Conclusion and Future Perspective of CRISPR-Cas systems

For patients and health professionals more rapid and more early diagnosis is very important especially for infectious diseases. CRISPR-Cas based diagnosis systems are able to provide the needed faster and accurate diagnosis. Also, conventional methods need sophisticated equipment and skilled or experienced personal while CRISPR-Cas based systems need less of these. Another advantage of the CRISPR-Cas over conventional systems is lower cost of the method. In their 2018 dated paper Lokko et al.[33] reported that economic value of in-vitro clinical diagnosis (IVD) will reach to $75 billion US Dollar by 2020. CRISPR-Cas based systems have the potential to lower this cost. Also, early diagnosis has the potential to reduce death ratio from infectious diseases. Considering all the recent data about conventional diagnostic methods, CRISPR-Cas based diagnostic tests have the potential for save lives from infectious disease patients with early and accurate diagnosis with a reduced cost.

As a conclusion, the CRISPR-Cas technology provides a diagnostic tool for pathogens and diseases. This system

offers low cost, fast laboratory process and high precision. Although CRISPR-Cas system is on the development stage, in the future it has the potential of being one the most used methods in the diagnostic laboratories.

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