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ISSN 2687-640X

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December 2022 Issue | ASOS
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ISSN:2687-640X
www.genediting.net

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Gene Editing Journal

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Review Article

©Gene Editing
(2022) 03: Pages 1-8.
DOI: 10.29228/genediting.63292

Gene editing tools and the treatment of cystic fibrosis disease

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Received: 28.06.2022 **Accepted/Published Online**: 26.10.2022 **Final Version**: 26.12.2022

Abstract: Gene therapy is an effective method for a wide range of genetic diseases, one of which is cystic fibrosis (CF) caused by gene mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The present study aimed to review the tools and studies focusing on the gene-editing of CF disease. Four main groups of endonuclease are currently used for genome editing, including meganucleases, zinc finger nucleases (ZFN), clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, and transcription activator-like effector nucleases (TALEN). The use of these genome editing techniques has converted the promise of a therapeutic approach for this disease to an achievable goal. Nevertheless, these techniques have so far only been tested on stem cells and have not yet been used in clinical treatments, due to the multifaceted characteristics of cystic fibrosis. Certainly, further advances in these techniques with increased efficiency of DNA cleavage sites and strong optimization to reduce the defects of their carriers direct the future of genomic editing technology to in vivo treatment of cystic fibrosis.

Key words: Gene editing; Cystic fibrosis; Endonuclease; DNA cleavage

1. Introduction

Nowadays, the development of efficient and reliable methods to make targeted and accurate changes, called genome editing, in the genome of living cells is one of the important goals of researchers in the field of biotechnology (Crane, et al. 2015; Doudna 2020). Gene therapy is an effective method for a wide range of genetic diseases caused by gene mutations (Marcaida, et al. 2010; Zhang 2021). In recent decades, virus-mediated methods have been developed for the efficient delivery of foreign genes to the human genome for the treatment of genetic diseases (Giacca and Zacchigna 2012; Xu, et al. 2021). Despite interesting results, this technology often had problems that limited its use, including the random integration of a foreign gene into the mammalian genome, the destruction of certain genes within the host, and the occurrence of unpredictable phenotypes (Check 2002; Huang, et al. 2021). In targeted genome editing techniques, accurate detection of the target site is important and DNA double-strand breaks are generated using engineered endonucleases at a predetermined locus (Ray and Raghavan 2020). The final repair of these breaks can be carried out by homologous recombination or non-homologous end joining (NHEJ) (Ensminger and Löbrich 2020). Framework mutations can occur in NHEJ due to insertions or deletions, while nucleotide exchange between an internal genomic region and a foreign DNA fragment, surrounded by homologous sequences, can be mediated by homologous recombination which manage a particular sequence through deletion, substitution, and insertion (Pauwels, et al. 2014).

An engineered endonuclease must have two important characteristics to be used in genome editing: 1) easy identification and cleavage of the DNA sequence, 2) specific detection of target DNA with a long sequence to prevent toxicity resulting from off-target genetic modification (Bao, et al. 2020). Based on these characteristics, four main groups of endonuclease are currently used for genome editing, including meganucleases, zinc finger nucleases (ZFN), clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, and transcription activator-like effector nucleases (TALEN) (Kaur, et al. 2021).
Despite its specificity, endonuclease can target 1 to 1,000 cells. To do better genome editing, powerful techniques are being presented that can be used to make genome editing in in-vivo models with genetic disorders. In this case, the first study was performed on mice with cystic fibrosis (CF), in which a whole exon was removed or more precisely, 3-bp (equivalent to the most common CF-causing mutation, ΔF508) was deleted (Harrison, et al. 2016). This study has attracted the attention of many geneticists to treat the disease through gene editing.
The present study aimed to introduce CF disease and review the research that has been performed on the genetic modification of this disease for therapeutic purposes.

2. Cystic fibrosis

Cystic fibrosis (CF) is one of the most prevalent lethal chronic genetic and multisystem disorders which is caused by abnormal transport of chloride ions across the apical membrane of

epithelial cells. The protein involved in this disease is called cystic fibrosis transmembrane conductance regulator (CFTR), is present on the cell membrane of many tissues, and plays an important role in regulating chloride ion transport. This protein channel in the apical membrane of airway wall cells undergoes conformational changes by ATP hydrolysis and cAMP-dependent phosphorylation, leading to the entrance of chlorine ions to the air lumen (Matsumoto, et al. 2021) (Figure 1).

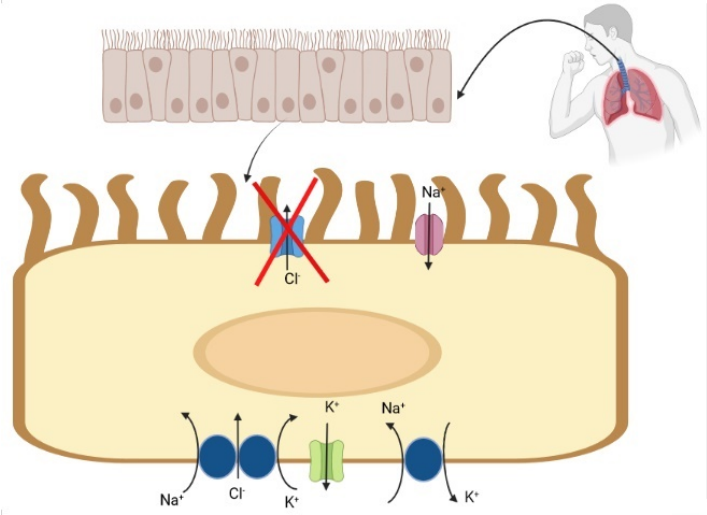

Figure. 1. The occurrence of dysfunction in the CFTR due to its gene mutation

Excessive mutations in the CFTR gene reduce the function of this protein and lead to the phenotype of cystic fibrosis (Iafusco, et al. 2021). In homozygous patients, the CFTR protein completely loses its function, thus increasing the viscosity of mucous secretions in the airways, pancreas, liver, vas deferens, and intestines. Of course, the most damage is to the respiratory and digestive systems (Gibson-Corley and Engelhardt 2021). Excess mucosa in the respiratory system of patients causes chronic infections and inability to breathe, which is the main cause of death in these patients. Most of these patients are unable to make pancreatic digestive enzymes due to pancreatic insufficiency and their life expectancy is very low (Verma, et al. 2020).

2.1. Genetics of cystic fibrosis and its geographical distribution

The CFTR gene has 27 exons and occupies an area of 250 kb on the long arm of chromosome 7 (7q31.2) (Gerbrands, et al. 2017). The promoter of this gene is rich in two nucleotides (guanine and cytosine) and the number of copies in different cells varies. Although more than 1,800 mutations have been reported in the CFTR gene, most of these mutations occur at position 508 of the CFTR protein (phe508del or ΔF508) (Çolak, et al. 2020) (Figure 2). The phe508del mutation accounts for 66% of CFTR mutations in many communities (Pineau, et al. 2021). It is not clear why the phe508del mutation is so common, but possibly because all phe508del alleles originated from an early genetic event that occurred at an early point in the human population drift (more than 10,000 years ago) (Callebaut, et al. 2020). The distribution of this mutation decreases from northwestern Europe to southeastern and western Asia, indicating the fundamental effect of this mutation. This downward slope continues from west to East Asia. However, the frequency of this mutation in West Asian countries is less than in European countries (13). Although the phe508del mutation is one of the most common CFTR mutations, the frequency of this mutation is low in the Middle East (53-49). In Iran, the most common mutations in CFTR protein are p.F508del, c.1677delTA, 2184delAAinsG, c.2183_2184delAAinsG, p.N1303K, c.2789 + 5G> A, p.S466X and p.G542X, respectively. These mutations are seen in Iran as compound heterozygotes (Banjar, et al. 2019; Khalilzadeh, et al. 2018).

3. Meganucleases and targeted gene editing

Meganucleases recognize long sequences of 14-40 bp in DNA that can be suitable targets for making double-stranded fragments in chromosomes and targeted genome engineering (Belete 2021). Among meganucleases, the LAGLIDADG family shows the most specificity in recognizing DNA sequences and consequently has been the most studied (Chevalier, et al. 2005). The genes encoding the LAGLIDADG family (homing endonucleases) are mostly located within introns and are classified as self-replicating genetic elements, although their role in the host is not yet well understood (Megarioti and Kouvelis 2020). The most important members of this family are I-CreI and PI-SceI (Moure, et al. 2002), which form the antiparallel β-sheet secondary structure and act as the DNA recognition unit. They form a saddle-shaped structure on the large groove of the DNA helix and are then catalyzed by the binding of divalent metal cations to the active site of the enzyme (Arnould, et al. 2011).

Some meganucleases form a heterodimer to recognize palindromic and quasi-palindromic target sites. Others are monomeric and recognize only non-palindromic sequences. Although these amazing sequences lead to specific recognition and less cytotoxicity than other gene-editing methods, some factors restrict the proper and effective use of meganucleases in gene editing including 1) complex and unknown interactions between DNA and these meganucleases; 2) DNA methylation in CpG sites, which reduces the enzyme's tendency to bind to DNA; 3) There is only one recognition site in the genome for each enzyme (18). The last problem can be solved by creating engineered meganucleases I) altering the amino acid sequences of meganuclease, II) fusing protein domains from different meganucleases, and III) creating chimeric meganuclease (Valton, et al. 2012).

3.1. Use of meganuclease in the treatment of cystic fibrosis

The design of engineered meganucleases that can be effective in the treatment of cystic fibrosis is under study, both in vivo and in vitro. These meganucleases can be considered to treat this disease by specifically recognizing abnormal mutations (Figure 2). In the genetic modification of cystic fibrosis, the target sequences around the ΔF508 mutation are recognized using engineered I-SceI meganuclease. Natural pTrc-ISceI, along with p11-LacY-S4 bound to the plasmid, which is likely to direct endonuclease, corrects the mutated sequence. Based on data obtained from I-SceI protein crystallization in the cystic fibrosis gene editing, 13 to 16 bp of DNA remain unbound after meganuclease binding (Chen, et al. 2009).

4. Zinc finger nucleases in gene editing

Zinc finger nucleases are synthetic proteins consisting of a DNA recognition domain of a zinc-finger transcription factor and a cleavage domain of FokI (Figure 2). Functional zinc finger nucleases are formed when the cleavage domains of FokI are close together and in good orientation, because the FokI domain acts only after forming a dimer (Carroll 2021).

There are several methods to develop zinc finger proteins for a given target sequence. One way is the modular assembly method, in which fingers for each component triplet are generated and then linked into a multi-finger peptide targeted to the corresponding composite sequence (Kaur, et al. 2021). The second way is to make finger proteins based on the OPEN (Oligomerized pool engineering) method that uses bacterial selections to identify finger combinations that will work well together. The third method is an OPEN-like system that uses a bacterial selection system that is similar to OPEN but with a different strategy for library construction (RASOOL 2021).

On the other hand, there are several strategies to minimize cleavage events at undesired locations including a) the design of zinc finger nucleases which are active only as a heterodimer, b) the use of spacer lengths from 4-6 bp, c) the use of variants that increase the catalytic activity of FokI, d) inactivating the catalytic activity of one ZFN monomer in the ZFN dimer required for double-strand cleavage (ZFNikases) (Urnov, et al. 2010).

Although the use of zinc finger proteins in gene editing is more advanced and useful than meganucleases due to its specificity, leading to its use in genomic editing of a wide range of cells and organisms, there are two major limitations. First, the zinc finger domain has a limited capacity to target the desired sequence of DNA due to the effects of DNA-binding proteins. Second, the absence of some zinc finger domains can cause unpredictable mutations and phenotypes by cleavage events at undesired locations, resulting in high cytotoxicity (Swarthout, et al. 2011).

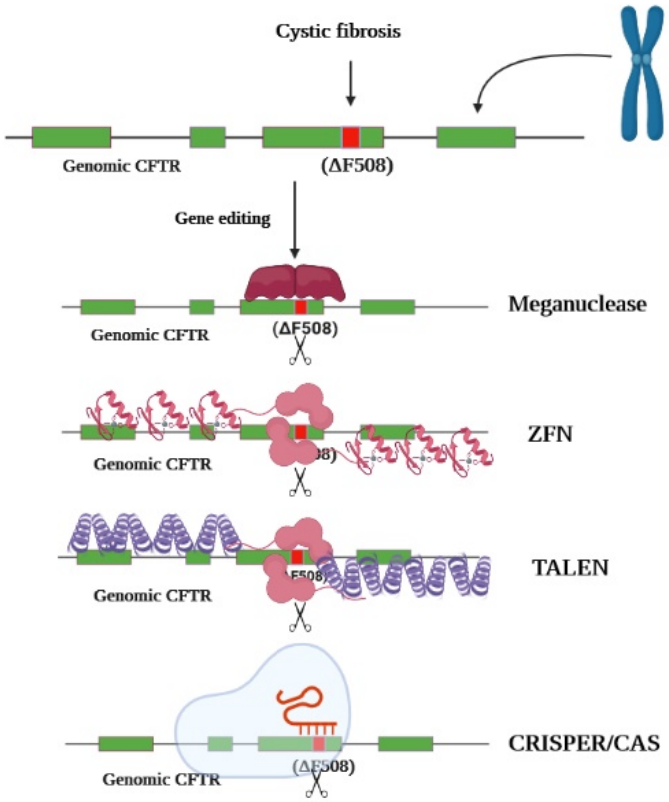

Figure. 2. Various methods for gene editing in cystic fibrosis

4.1. Use of zinc finger proteins in the treatment of cystic fibrosis

The use of the OPEN technique to edit mutations in CFTR protein was reported. However, it has low potential in gene editing. This low editing potential of ZFN in the CFTR gene may be attributed to the difficult access of ZNFs to DNA due to chromatin effects or methylation of target sites (Lee, et al. 2012). A recent study used the Modular assembly method to design ZFNs that recognize the 18-bp target site on the intron 9 of human CFTR sequences (203 bp upstream of the ΔF508 lesion was deleted). This study showed that ΔF508 mutation can be edited by this method. It was suggested that ZFNs are more effective in treating cystic fibrosis if the cDNA minigene will be accompanied by a suitable viral vector (Harrison, et al. 2016; Lee, et al. 2012).

Small molecule treatments targeting CFTR are only effective for a small percentage of individuals with specific CFTR mutations. To overcome this limitation, recently, Villamizar et al. (2021) engineered stromal-derived mesenchymal stem cells (MSC) and HEK293 cells to produce exosomes containing a novel CFTR Zinc Finger Protein fusion with transcriptional activation domains VP64, P65, and Rta to target the CFTR promoter (CFZF-VPR) and activate transcription. Results showed the activation of CFTR transcription in patient-derived Human Bronchial Epithelial cells (HuBEC). Moreover, MSC-derived

exosomes can be used to deliver a packaged zinc finger activator to target cells and activate CFTR. In other words, they presented a novel method that has the potential to be used in next-generation genetic therapy and may be effective in treating patients afflicted with cystic fibrosis (Villamizar, et al. 2021).

5. Genome editing using transcription activator-like effector nucleases (TALEN)

The TALEN gene-editing technique emerged after zinc finger proteins. Like ZFN, chimeric proteins are used in the TALEN method. Components of this method include the transcription activator-like effector (TALE) DNA binding domain and DNA cleavage domain (FokI) (Barnett 2018) (Figure 2). TALEs are proteins secreted by the bacteria and help them to bind to sequences in the plant host genome (Richter, et al. 2020). The DNA binder domain has repeated 33-35 amino acid sequences, in which the 12th and 13th amino acids are called the Repeat Variable Diresidue (RVD), are highly variable, and able to recognize a nucleotide (Malzahn and Qi 2021). Unlike ZFN, this method is made easily and quickly for any DNA sequence, produces more specific targeting, and has much less cytotoxicity. It is therefore more useful for gene editing (Ding, et al. 2013).

In the optimal TALEN structure with the highest efficiency, a 127 amino acid sequence is located just before the central replication units, which is critical for the stable binding of the central part to DNA. The FokI cleavage domains must form a heterodimer structure at the cleavage site to be activated (Gao, et al. 2012). TALEN homodimers have a high ability to create non-specific cleavage, which reduces their safety and efficiency in genome editing. Therefore, the mutation is induced in the cleavage domain to prevent the formation of homodimers (Aouida, et al. 2014).

Designing an efficient TALEN requires at least 3 to 4 strong RVDs. More than 20 different RVDs have been identified in TALENs, the most common of which are RG, HD, NN, KN recognizing the nucleotides T, C, G, G, respectively. The lack of a strong specific RVD for guanine limits the widespread use of TALENs. Further studies have shown that RVD NH is both highly specific for guanine base and more efficient than RVD NK (Yang, et al. 2014).

Since TALEN repeat arrays are so similar, it is very difficult to design plasmids encoding a long sequence of TALEN repeat units. Several methods were introduced to solve this problem, one of which is the REAL (Restriction Enzyme and Ligation) method. The REAL method uses restriction enzyme (II) to connect the single units of TALEN repeat arrays (Sakuma, et al. 2013). Another method used is the Golden gate cloning-based assembly, in which assembling the repetitive units and binding the arrays is much simpler and faster than the previous method (Beikzadeh and Angaji 2019). The fast ligation-based automatable solid-phase high throughput (FLASH) method is similar to the REAL method. However, there is only one exception, namely the connection of repeating units is carried out on magnetic (Reyon, et al. 2013).

5.1. TALEN gene editing and treatment of cystic fibrosis

The use of the TALEN tool to edit the human CFTR gene has been performed based on a yeast reporter but has not yet been fully tested in human cells (Harrison, et al. 2016; Kormann 2015). A previous study on the gene-editing of epithelial cells in people with cystic fibrosis showed that the CFTR gene can be edited by the FLASH method and using wtCFTR-SDFs (plasmids carrying no gene). It was also found that cystic fibrosis-induced pluripotent stem cells (CF-iPSCs) can be differentiated after the editing, and their cAMP-activated Cl^- channel had a function similar to that of normal epithelial cell pumps (Suzuki, et al. 2016).

After that, Xia et al. (2019) used TALEN-mediated gene targeting for cystic fibrosis gene therapy. They engineered helper-dependent adenoviral (HD-Ad) vectors to deliver a pair of TALENs together with donor DNA targeting the human AAVS1 locus. Their study showed precise insertion of human CFTR minigene into the target site. The efficiency of gene integration was about 5% and the CFTR vector transduced cells had CFTR mRNA expression. Generally, their findings indicated a new direction for future in vitro and in vivo studies in CF gene editing (Xia, et al. 2019).

Fleischer et al. (2020) repaired the CFTR activity of iPSC-derived intestinal organoids from cystic fibrosis patients using the TALEN tool. Despite describing more than 2,000 mutations in the *CFTR* gene, mutation at amino acid position 508 (p.F508del) is responsible for the vast majority of CF cases worldwide. They tried to use genome-editing techniques to restore CFTR activity in p.F508del patient-derived induced pluripotent stem cells (iPSCs). They found that the seamless restoration of the p.F508del mutation resulted in the normal expression of the mature CFTR glycoprotein, full recovery of CFTR activity, and normal response of the repaired organoids to treatment with two approved CF therapies: VX-770 and VX-809 (Fleischer, et al. 2020).

6. CRISPR/Cas system

Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nuclease (CRISPR/Cas) was a new technology to rapidly and efficiently edit the gene, emerged in 2015 as a revolution in biological science, especially medicine. CRISPR/Cas 9 is a type of bacterial endonuclease that specifically binds to a 20 bp sequence (instead of binding to DNA-binding

proteins) and travels to the target DNA. CRISPER was first discovered in 1978 in *E. coli* and the term CRISPR was first used in 2002 to describe repeat sequences in the bacterial genome (Munawar and Ahmad 2021). The importance of these sequences became clear when the researchers realized the similarity of the phage genome sequences with the sequences in the CRISPR locus (Horvath, et al. 2009). In the same years, it was discovered that viruses are unable to infect archaebacteria with CRISPR sequences. This finding further revealed that the CRISPR system is considered a memory storage unit and defense mechanism against bacteriophage infection (Shipman, et al. 2017).

In the CRISPR/Cas system, RNA-guided nucleases are used to cut foreign genetic elements (Figure 2). Three types of CRISPR/Cas systems have been identified in bacteria and archaebacteria, all of which have CRISPR RNA (crRNA), consisting of a set of CRISPR-related genes, the Cas enzyme, non-coding RNAs, a separate sequence of repeated elements with short sequences (protospacers) derived from foreign DNA (Reimann, et al. 2020). Within the target DNA, each protospacer always has an adjacent motif called PAM (Adjacent motif protospacer), which can be varied according to the proprietary CRISPR system. The second type of CRISPR system requires trans-activating CRISPR RNA (tracrRNA), crRNA, and cas9 (CRISPR-related protein) to make double-strand breaks in the target DNA. Making targeted cleavages by Cas9 requires specific interactions between crRNA and tracrRNA, as well as the establishment of base pairs between crRNA and 20 nucleotides of the target DNA. Downstream of the target sequence, there is a three-nucleotide (as NGG) short motif (called PAM), which plays a very important role in the identification of the target sequence by Cas9 (Zhang, et al. 2021). The Cas9 nuclease makes cleavage in target DNA about three bp upstream of PAM (Anders, et al. 2014). In targeted genome engineering, crRNA and tracrRNA form a chimeric RNA called gRNA (guide RNA), which can mimic the function of both RNAs by creating a series hairpin structure. Cas9 nucleases make specific breaks using HNH and RuvC-protected nuclease domains. Variants of Cas9, called Cas9n (Cas9 Nikase), can be created by mutating one of these domains, that can only cut one of the two strands of DNA (Ye, et al. 2021).

CRISP/Cas9 technology has more advantages than other gene-editing technologies such as TALEN and ZFN. These preferences are 1) the Cas9 cleavage leads to much more varied fragments, 2) it is enough to use only a special gRNA sequence for a new sequence to target it, and 3) designing various gRNAs in CRISPR/Cas9 technology provides the possibility of targeting several editing sites simultaneously (Gaj, et al. 2013).

6.1. Use of CRISPR/Cas9 gene editing in the treatment of cystic fibrosis

The use of the CRISPR/Cas9 tool in the treatment of cystic fibrosis was performed for the first time in the intestinal stem cells of adult mice in 2013. In this experiment, after culturing intestinal stem cells, trypsinization, and preparing cell suspension, they were transfected using lipofectamine, a plasmid encoding Cas9, and sgRNA to the target DNA of intestinal epithelial stem cells. In this experiment, only Lgr5+ stem cells were able to grow and form colonies, and other epithelial cells did not. Two weeks after seeding of transfected cells, 5 different phenotypes of mutations, resulting from sgRNA transfection, appeared and do not show the morphological features of cystic fibrosis. (Schwank, et al. 2013).

Subsequent studies have developed the use of CRISPR/Cas9 technology in human intestinal epithelial stem cells. The difference was that the transfected cells were cultured in Wnt- and R-spondin-free medium. The development of studies on this technology has led to the development of CRISPR/Cas9 for the genome editing of intestinal stem cells in humans with cystic fibrosis. During these studies, adult intestinal epithelial stem cells were transfected with two different types of sgRNAs (that target the exon or intron 11 of CFTR genes and transported by the plasmid) using CRISPR/Cas9, followed by the F508 mutation editing.

Ruan et al (2019) evaluated efficient gene editing at major CFTR mutation loci. They compared different transfection methods (lipofectamine versus electroporation) and formats (plasmid DNA versus ribonucleoprotein) in delivering the CRISPR/Cas9 elements along with single-stranded oligodeoxynucleotides (ssODNs) to clinically relevant cells targeting major CFTR mutation loci. Their findings showed that among different combinations, electroporation of CRISPR/Cas9 and guide RNA (gRNA) ribonucleoprotein (Cas9 RNP) is the most effective strategy, leading to achieving a greater than 20% precise correction rate when it is applied to a patient-derived iPSC line carrying the dF508 mutation (Ruan, et al. 2019). On the other hand, Smirnikhina et al. (2020) tested P.F508del editing in cells from cystic fibrosis patients. Their findings showed that CRISPR/Cas9 can correct p.F508del mutation in the *CFTR* gene in the CFTE29o- cells and induced pluripotent stem cells (iPSCs) derived from patients with cystic fibrosis (CF). However, p.F508del mutation editing using CRISPR/Cas9 in CF patient-derived iPSCs was a relatively rare event and subsequent cell selection and cultivation should be carried out (Smirnikhina, et al. 2020).

Vaidyanathan et al. (2021) tested the targeted replacement of full-length CFTR in human airway stem cells by CRISPR/Cas9 for pan-mutation correction in the endogenous locus. They used CRISPR/Cas9 and two adeno-associated viruses (AAV) carrying the two halves of the *CFTR* cDNA to sequentially insert the full

CFTR cDNA along with a truncated CD19 (tCD19) enrichment tag in upper airway basal stem cells (UABCs) and human bronchial basal stem cells (HBECs). These engineered and differentiated cells showed restored CFTR function that was >70% of the CFTR function in non-CF controls. Thus, this study was able to develop a therapy for almost all CF patients, including patients who cannot be treated using recently approved modulator therapies (Vaidyanathan, et al. 2021). A recent study tried to prove the better performance of CRISPR/Cas9 in editing CFTR mutation. Santos et al. (2022) compared the effectiveness of Cas9 and Cas12a CRISPR editing methods to correct W1282X-CFTR mutation. They found that Cas9 showed higher levels of correction than Cas12a (Santos, et al. 2022). New studies more used CRISPR/CAS9 for editing CFTR mutation (Yan, et al. 2022).

7. Conclusions and future prospects

The cloning of the CFTR gene has been carried out more than two decades ago and many technologies have been developed to clinically identify this genetic defect and target defective cells and remove mutations. Nowadays, new treatments for this disease, instead of diagnosing the symptoms of the disease, aim to eliminate the genetic cause of the cystic fibrosis phenotype. Many of these technologies use a variety of strategies, including overcoming patients with CFTR dysfunction, the use of the functional transcript of CFTR protein, and direct editing of genomic mutations promise clinical treatment for these patients. Meanwhile, the use of genome editing techniques such as meganucleases, ZFNs, TALENs, and the CRISPR/Cas9 system has converted the promise of a therapeutic approach for this disease into an achievable goal. Nevertheless, these techniques have so far only been tested on stem cells and have not yet been used in clinical treatments, because cystic fibrosis is a multifaceted disease that makes it difficult to treat with current knowledge of gene editing. Certainly, further advances in these techniques with increased efficiency of DNA cleavage sites and strong optimization to reduce the defects of their carriers direct the future of genomic editing technology to in vivo treatment of cystic fibrosis.

Acknowledgments: The author would like to thank faculty members of cellular and molecular biology in Shahr-e-Qods Branch of Islamic Azad University for their scientific guidance.

Conflicts of interest: there is no conflicts of interest.

References

Anders C, Niewoehner O, Duerst A, Jinek M (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513:569-573

Aouida M, Piatek MJ, Bangarusamy DK, Mahfouz MM (2014). Activities and specificities of homodimeric TALENs in Saccharomyces cerevisiae. Current genetics 60:61-74

Arnould S, Delenda C, Grizot S, Desseaux C, Paques F, Silva G, Smith J (2011). The I-CreI meganuclease and its engineered derivatives: applications from cell modification to gene therapy. Protein Engineering, Design & Selection 24:27-31

Banjar H, Al-Mogarri I, Nizami I, Al-Haider S, AlMaghamsi T, Alkaf S, Al-Enazi A, Moghrabi N (2019). Geographic distribution of cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations in Saudi Arabia. International Journal of Pediatrics and Adolescent Medicine

Bao XR, Pan Y, Lee CM, Davis TH, Bao G (2020). Tools for experimental and computational analyses of off-target editing by programmable nucleases. Nature protocols 1-17

Barnett P (2018). Transcription activator like effector nucleases (TALENs): a new, important, and versatile gene editing technique with a growing literature. Science & Technology Libraries 37:100-112

Beikzadeh B, Angaji S (2019). TALEN: A Tool for Genome Editing.

Belete TM (2021). The Current Status of Gene Therapy for the Treatment of Cancer. Biologics: Targets & Therapy 15:67

Callebaut I, Mense M, Farinha CM (2020) Exploring the basic mechanisms in Cystic Fibrosis: Promoting data presentation and discussion at the 16th ECFS Basic Science Conference. In. Elsevier

Carroll D (2021). A Short, Idiosyncratic History of Genome Editing. Gene and Genome Editing 100002

Check E (2002) A tragic setback. In. Nature Publishing Group

Chen Z, Wen F, Sun N, Zhao H (2009). Directed evolution of homing endonuclease I-SceI with altered sequence specificity. Protein Engineering, Design & Selection 22:249-256

Chevalier B, Monnat RJ, Stoddard BL (2005) The LAGLIDADG homing endonuclease family. In: Homing endonucleases and inteins. Springer, pp 33-47

Çolak Y, Nordestgaard BG, Afzal S (2020). Morbidity and mortality in carriers of the cystic fibrosis mutation CFTR Phe508del in the general population. European Respiratory Journal 56:

Crane AM, Kramer P, Bui JH, Chung WJ, Li XS, Gonzalez-Garay ML, Hawkins F, Liao W, Mora D, Choi S (2015). Targeted correction and restored function of the CFTR gene in cystic fibrosis induced pluripotent stem cells. Stem cell reports 4:569-577

Ding Q, Lee Y-K, Schaefer EA, Peters DT, Veres A, Kim K, Kuperwasser N, Motola DL, Meissner TB, Hendriks WT (2013). A TALEN genome-editing system for generating human stem cell-based disease models. Cell stem cell 12:238-251

Doudna JA (2020). The promise and challenge of therapeutic genome editing. Nature 578:229-236

Ensminger M, Löbrich M (2020). One end to rule them all: non-homologous end-joining and homologous recombination at DNA double-strand breaks. The British journal of radiology 93:20191054

Fleischer A, Vallejo-Díez S, Martín-Fernández JM, Sánchez-Gilabert A, Castresana M, Del Pozo A, Esquisabel A, Ávila S, Castrillo JL, Gaínza E (2020). iPSC-derived intestinal organoids from cystic fibrosis patients acquire CFTR activity upon TALEN-

mediated repair of the p. F508del mutation. Molecular Therapy-Methods & Clinical Development 17:858-870

Gaj T, Gersbach CA, Barbas III CF (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in biotechnology 31:397-405

Gao H, Wu X, Chai J, Han Z (2012). Crystal structure of a TALE protein reveals an extended N-terminal DNA binding region. Cell research 22:1716-1720

Gerbrands LC, Haarman EG, Hankel MA, Finken MJ (2017). Cystic fibrosis and Silver–Russell syndrome due to a partial maternal isodisomy of chromosome 7. Clinical case reports 5:1697

Giacca M, Zacchigna S (2012). Virus-mediated gene delivery for human gene therapy. Journal of controlled release 161:377-388

Gibson-Corley KN, Engelhardt JF (2021). Animal Models and Their Role in Understanding the Pathophysiology of Cystic Fibrosis–Associated Gastrointestinal Lesions. Annual Review of Pathology: Mechanisms of Disease 16:51-67

Harrison PT, Sanz DJ, Hollywood JA (2016). Impact of gene editing on the study of cystic fibrosis. Human genetics 135:983-992

Horvath P, Coûté-Monvoisin A-C, Romero DA, Boyaval P, Fremaux C, Barrangou R (2009). Comparative analysis of CRISPR loci in lactic acid bacteria genomes. International journal of food microbiology 131:62-70

Huang L, Wan J, Wu Y, Tian Y, Yao Y, Yao S, Ji X, Wang S, Su Z, Xu H (2021). Challenges in adeno-associated virus-based treatment of central nervous system diseases through systemic injection. Life Sciences 119142

Iafusco F, Maione G, Rosanio FM, Mozzillo E, Franzese A, Tinto N (2021). Cystic Fibrosis-Related Diabetes (CFRD): Overview of Associated Genetic Factors. Diagnostics 11:572

Kaur G, Upadhyay SK, Verma PC (2021). An Overview of Genome-Engineering Methods. Genome Engineering for Crop Improvement 1-21

Khalilzadeh S, Hassanzad M, Toutkaboni MP, Nejad ST, Sheikholeslami F-M, Velayati AA (2018). Reappraisal of frequency of common cystic fibrosis transmembrane conductance regulator gene mutations in Iranian cystic fibrosis patients. Tanaffos 17:73

Kormann MSD (2015) In vivo gene correction of cystic fibrosis. In: Cystic Fibrosis in the Light of New Research. IntechOpen

Lee CM, Flynn R, Hollywood JA, Scallan MF, Harrison PT (2012). Correction of the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator gene by zinc-finger nuclease homology-directed repair. BioResearch open access 1:99-108

Malzahn AA, Qi Y (2021) Assembly of TALEN and mTALE-Act for Plant Genome Engineering. In: Crop Breeding. Springer, pp 207-218

Marcaida MJ, Munoz IG, Blanco FJ, Prieto J, Montoya G (2010). Homing endonucleases: from basics to therapeutic applications. Cellular and Molecular Life Sciences 67:727-748

Matsumoto Y, Shiozaki A, Kosuga T, Kudou M, Shimizu H, Arita T, Konishi H, Komatsu S, Kubota T, Fujiwara H (2021). Expression and role of CFTR in human esophageal squamous cell carcinoma. Annals of surgical oncology 1-13

Megarioti AH, Kouvelis VN (2020). The coevolution of fungal mitochondrial introns and their Homing Endonucleases (GIY-YIG and LAGLIDADG). Genome biology and evolution 12:1337-1354

Moure CM, Gimble FS, Quiocho FA (2002). Crystal structure of the intein homing endonuclease PI-Sce I bound to its recognition sequence. Nature structural biology 9:764-770

Munawar N, Ahmad A (2021) CRISPR/Cas System: An Introduction. In: CRISPR Crops. Springer, pp 1-35

Pauwels K, Podevin N, Breyer D, Carroll D, Herman P (2014). Engineering nucleases for gene targeting: safety and regulatory considerations. New biotechnology 31:18-27

Pineau F, Caimmi D, Taviaux S, Reveil M, Brosseau L, Rivals I, Drevait M, Vachier I, Claustres M, Chiron R (2021). DNA Methylation at ATP11A cg11702988 Is a Biomarker of Lung Disease Severity in Cystic Fibrosis: A Longitudinal Study. Genes 12:441

RASOOL N (2021). NEW PLANT BREEDING TECHNIQUES: PROBLEMS AND PROSPECTS. Mutagenesis, Cytotoxicity and Crop Improvement: Revolutionizing Food Science 66

Ray U, Raghavan SC (2020). Modulation of DNA double-strand break repair as a strategy to improve precise genome editing. Oncogene 39:6393-6405

Reimann V, Ziemann M, Li H, Zhu T, Behler J, Lu X, Hess WR (2020). Specificities and functional coordination between the two Cas6 maturation endonucleases in Anabaena sp. PCC 7120 assign orphan CRISPR arrays to three groups. RNA biology 17:1442-1453

Reyon D, Maeder ML, Khayter C, Tsai SQ, Foley JE, Sander JD, Joung JK (2013). Engineering Customized TALE Nucleases (TALENs) and TALE Transcription Factors by Fast Ligation-Based Automatable Solid-Phase High-Throughput (FLASH) Assembly. Current protocols in molecular biology 103:12.16. 11-12.16. 18

Richter I, Uzum Z, Stanley CE, Moebius N, Stinear TP, Pidot SS, Ferling I, Hillmann F, Hertweck C (2020). Secreted TAL effectors protect symbiotic bacteria from entrapment within fungal hyphae. bioRxiv

Ruan J, Hirai H, Yang D, Ma L, Hou X, Jiang H, Wei H, Rajagopalan C, Mou H, Wang G (2019). Efficient gene editing at major CFTR mutation loci. Molecular Therapy-Nucleic Acids 16:73-81

Sakuma T, Hosoi S, Woltjen K, Suzuki Ki, Kashiwagi K, Wada H, Ochiai H, Miyamoto T, Kawai N, Sasakura Y (2013). Efficient TALEN construction and evaluation methods for human cell and animal applications. Genes to Cells 18:315-326

Santos L, Mention K, Cavusoglu-Doran K, Sanz DJ, Bacalhau M, Lopes-Pacheco M, Harrison PT, Farinha CM (2022). Comparison of Cas9 and Cas12a CRISPR editing methods to correct the W1282X-CFTR mutation. Journal of Cystic Fibrosis 21:181-187

Schwank G, Koo B-K, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, van der Ent CK (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell stem cell 13:653-658

Shipman SL, Nivala J, Macklis JD, Church GM (2017). CRISPR–Cas encoding of a digital movie into the genomes of a population of living bacteria. Nature 547:345-349

Smirnikhina SA, Kondrateva EV, Adilgereeva EP, Anuchina AA, Zaynitdinova MI, Slesarenko YS, Ershova AS, Ustinov KD,

Yasinovsky MI, Amelina EL (2020). P. F508del editing in cells from cystic fibrosis patients. Plos one 15:e0242094

Suzuki S, Sargent RG, Illek B, Fischer H, Esmaeili-Shandiz A, Yezzi MJ, Lee A, Yang Y, Kim S, Renz P (2016). TALENs facilitate single-step seamless SDF correction of F508del CFTR in airway epithelial submucosal gland cell-derived CF-iPSCs. Molecular Therapy-Nucleic Acids 5:e273

Swarthout JT, Raisinghani M, Cui X (2011). Zinc Finger Nucleases: A new era for transgenic animals. Annals of neurosciences 18:25

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010). Genome editing with engineered zinc finger nucleases. Nature Reviews Genetics 11:636-646

Vaidyanathan S, Baik R, Chen L, Bravo DT, Suarez CJ, Abazari SM, Salahudeen AA, Dudek AM, Teran CA, Davis TH (2021). Targeted replacement of full-length CFTR in human airway stem cells by CRISPR/Cas9 for pan-mutation correction in the endogenous locus. Molecular Therapy

Valton J, Daboussi F, Leduc S, Molina R, Redondo P, Macmaster R, Montoya G, Duchateau P (2012). 5′-Cytosine-phosphoguanine (CpG) methylation impacts the activity of natural and engineered meganucleases. Journal of Biological Chemistry 287:30139-30150

Verma P, Vishwakarma VK, Paswan SK, Rao CV, Srivastava S (2020) Cystic Fibrosis: Biology and Therapeutics. In: Chronic Lung Diseases. Springer, pp 61-74

Villamizar O, Waters SA, Scott T, Grepo N, Jaffe A, Morris KV (2021). Mesenchymal Stem Cell exosome delivered Zinc Finger Protein activation of cystic fibrosis transmembrane conductance regulator. Journal of extracellular vesicles 10:e12053

Xia E, Zhang Y, Cao H, Li J, Duan R, Hu J (2019). TALEN-mediated gene targeting for cystic fibrosis-gene therapy. Genes 10:39

Xu X, Chen W, Zhu W, Chen J, Ma B, Ding J, Wang Z, Li Y, Wang Y, Zhang X (2021). Adeno-associated virus (AAV)-based gene therapy for glioblastoma. Cancer Cell International 21:1-10

Yan Z, Vorhies K, Feng Z, Park S-Y, Choi SH, Zhang Y, Winter M, Sun X, Engelhardt JF (2022). rAAV-MEDIATED EDITING OF THE G551D CFTR MUTATION IN FERRET AIRWAY BASAL CELLS. Human Gene Therapy

Yang J, Zhang Y, Yuan P, Zhou Y, Cai C, Ren Q, Wen D, Chu C, Qi H, Wei W (2014). Complete decoding of TAL effectors for DNA recognition. Cell research 24:628-631

Ye T, Duan Y, Tsang HW, Xu H, Chen Y, Cao H, Chen Y, Fu AK, Ip NY (2021). Efficient manipulation of gene dosage in human iPSCs using CRISPR/Cas9 nickases. Communications biology 4:1-10

Zhang B (2021). CRISPR/Cas gene therapy. Journal of Cellular Physiology 236:2459-2481

Zhang S, Shen J, Li D, Cheng Y (2021). Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. Theranostics 11:614

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Review Article

©Gene Editing
(2022) 03: Pages 9-13.
DOI: 10.29228/genediting.67056

Gene Therapy Products Approved in 2022

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Received: 14.12.2022 **Accepted/Published Online**: 21.12.2022 **Final Version**: 25.12.2022

Abstract: Cell and gene therapy is a method used to treat genetically based diseases that currently have no effective treatments. The number of products available for cell and gene therapy has been rising annually in recent years. By 2025, the FDA predicts that it will have approved 10 to 20 novel cell and gene therapy products annually. The FDA's current list of authorized gene treatments includes 26 gene and cell-based products. The FDA and EMA have approved 8 cell-based and gene therapy products in 2022. We summarized the gene therapy medications authorized in 2022 in this review. Two gene therapy drugs (Skysona and Zynteglo) that were approved this year were previously approved and stopped marketing in Europe since the European market price could not be agreed upon. This year, the FDA approved the use of these two medications.

Key words: gene editing, cell-based gene editing, gene editing products, genetic disease

1. Introduction

Gene therapy is a potential technique for creating treatments for conditions with genetic causes and those for which there is no known solution. Plasmids with the desired transgenes must be transfected into the target cells for gene therapy to be effective. Gene therapy is a method used in the treatment of diseases with a genetic basis and for diseases that currently have no cure [1]. The US Food and Drug Administration (FDA) describes gene therapy as the transmission of genetic material through transcription or translation and/or via nucleic acids, viruses, or genetically modified microorganisms that manifest their effects by integrating into the host genome [2]. When we look at the page of gene therapy products approved by the FDA, we see that currently 26 approved gene therapy products are listed [3]. In 2021, the EMA authorized Skysona for the treatment of early cerebral adrenoleukodystrophy (CALD) in patients who were age of eighteen or younger. Zynteglo is a gene therapy medicine that was authorized in 2019 for beta thalassemia [4]. This year, the FDA approved the use of these two gene therapy medicines. The gene therapy products listed here have been given FDA or EMA approval for application in 2022.

2. Gene Therapy Products

Hemgenix (Etranacogene dezaparvovec)

Hemophilia is a rare genetic disease caused by blood coagulation factor VIII and IX, a protein that helps blood clot to stop bleeding, deficiency. Hemophilia A and Hemophilia B are both brought on by factor VIII and factor IX deficiencies, respectively [5]. Hemophilia B affects approximately one in every 30,000 men worldwide. In the 1950s, it was considered different from hemophilia A and was named Christmas disease, taking the surname of the first affected family [6,7].

Hemgenix is an adeno-associated virus vector-based gene therapy containing a gene for coagulation factor IX, the active ingredient of which is Etranacogene dezaparvovec. The patient's liver produces factor IX protein as a result of this gene expression in the liver, which raises blood levels of factor IX and reduces the risk of bleeding incidents [8]. Etranacogene dezaparvovec, marketed as Hemgenix, is a one-time gene therapy administered by IV infusion, is a gene therapy used to treat hemophilia B. The FDA examined the safety and effectiveness of Hemgenix in two studies including 57 adult men with severe or moderate hemophilia B, aged 18 to 75. Its efficacy was determined based on reductions in annual bleeding rate (ABR) in men [9]. The study of 54 participants showed increases in patients' Factor IX activity levels and a 54% reduction in annual bleeding rate from baseline [10].

On November 22, 2022, the gene therapy product Hemgenix, produced to treat hemophilia B, was approved by the FDA [11]. That product is the first and only gene therapy treatment for hemophilia B disease. Following FDA approval, Pennsylvania-based pharmaceutical company CSL Behring LLC published its $3.5 million price tag. That price made Hemgenix the world's most expensive drug, surpassing Spinal muscular atrophy (SMA) gene therapy from Novartis, Zolgensma, which is also a single-dose drug, costing around $2 million per dose [12].

Roctavian (Valoctocogene roxaparvovec)

Hemophilia is a serious genetic disease with x-linked recessive inheritance in which blood coagulation is inadequate. Hemophilia is named for the deficiency of the factor it contains. Congenital deficiency of factor 8 protein is called hemophilia A, and deficiency of factor 9 protein is called hemophilia B. The incidence of hemophilia A is much higher than hemophilia B. Patients with hemophilia A either produce too little Factor VIII or produce a dysfunctional version of the protein [13]. The traditional treatment for hemophilia A is the administration of recombinant or plasma concentrated Factor VIII to the patient by regular intravenous infusions.

It is a gene therapy product for the treatment of hemophilia A, sold under the brand name Roctavian and whose active ingredient is valoctocogene roxaparvovec [14]. To replace the depleted blood clotting protein, Roctavian introduces functional factor VIII genes into liver cells using an adeno-associated virus type 5 (AAV5) vector. According to conventional treatments, patients with hemophilia A need to have one or more injections every week or month to simulate or replace the missing factor VIII. In patients with hemophilia A, Roctavian has been demonstrated to be beneficial in raising factor VIII activity, and this increase has been sustained for at least two years [15]. In contrast to approved Hemlibra medication, ICER's draft evidence report evaluated the lifetime cost per patient of treating hemophilia A with a single dosage of Roctavian. It calculated the annual cost of preventive Hemlibra treatments to be $640,000 as opposed to the $2.5 million one-time cost of Roctavian [16].

This gene therapy product was developed by BioMarin Pharmaceutical and received approval for medical use from the European Commission in August 2022 [16]. But also BioMarin International Limited withdrew its application for marketing authorization for Roctavian for the treatment of severe hemophilia A on November 4, 2020. In its letter, which it stated as the reason for the withdrawal, it stated that it had withdrawn its application because it could not provide the requested data during the current procedure. The company further added that it will continue to develop Roctavian for the treatment of hemophilia A and reserves the right to submit another application in the future [17]. This genetic medicine is important in that it is the first product for vast patient populations to offer prospective one-off therapies. This gene therapy product has not yet been approved by the FDA.

Upstraza (Eladocagene exuparvovec)

The brain is impacted by the uncommon genetic condition known as AADC deficiency, causing weak muscle tone and delaying a child's development. An alteration in the DDC gene, which lowers the activity of the AADC enzyme, is the genetic cause of AADC deficiency. The AADC enzyme is essential for making neurotransmitters. Therefore, a decrease in enzyme activity means a decrease in neurotransmitter levels. AADC deficiency is a disease that prevents cells in the brain from communicating by means of neurotransmitters [18,19].

A gene therapy product called Upstaza expresses the human AADC enzyme, whose active ingredient is Eladocagene exuparvovec. Adeno-associated virus type 2 (AAV2)-based vector with the human DDC gene's cDNA is used in this treatment [20]. The active biological ingredient of Upstraza is a recombinant AAV2 vector carrying the human DDC gene and cDNA [21]. Patients eighteen months of age and older with a clinically, molecularly, or genetically confirmed diagnosis of AADC deficiency with a severe phenotype may be treated with Upstraza. Stereotactic injections are used to provide this gene therapy treatment directly into the brain. By delivering a working DDC gene to the putamen, this product, a one-time therapy, corrects the underlying genetic abnormality. As a consequence, the AADC enzyme is produced, dopamine synthesis is restored, and motor function is improved [22].

On July 20, 2022, the European Commission authorized eladocagene exuparvovec for the treatment of AADC deficiency in individuals 18 months of age and older [23].

Breyanzi (Lisocabtagene maraleucel)

Lymphoma is a clonal malignant disease group that arises as a result of somatic mutation of a single lymphocyte progenitor cell and includes different subgroups. Lymphomas consist of T and B lymphocytes and rarely NK (natural killer) cell lines although they can happen in any organ of the body, lymph nodes are where they often start. Lymphomas are generally split into 2 large groups as Hodgkin and non-Hodgkin lymphomas (HDL) with their clinical and morphological features and treatment modalities [24]. Adults are most likely to develop the lymphoid neoplasm known as diffuse large B-cell non-Hodgkin lymphoma (DLBCL), with around 30,000 new cases identified globally each year. DLBCL is responsible for 40% of all non-Hodgkin lymphomas (NHL) [25].

Under the brand name Breyanzi, the medication is also referred to as Lisocabtagene maraleucel, a chimeric antigen receptor T cell (CAR-T) treatment that is used to treat large B-cell lymphoma. Breyanzi is an anti-CD19 chimeric antigen receptor-carrying replication-deficient lentiviral vector transduced ex vivo into purified CD8+ and CD4+ T cells. It is a CD19-directed genetically modified autologous cell-based product. Adult patients with relapsed or refractory DLBCL, primary mediastinal large B-cell lymphoma (PMBCL), and follicular lymphoma grade 3B are advised to use this gene therapy product (FL3B) [26].
Breyanzi includes lisocabtagene maraleucel, which combines the patient's two kinds of white blood cells (CD4+ and CD8+ T

cells). These T cells were genetically modified in the lab to generate the CAR protein. CAR may bind to the protein CD19, which is present on the surface of cancer cells. When a cancer patient receives this treatment, the patient's own T cells that modified T cells taken from patients connect to the CD19 proteins on the patient's cancer cells and destroy them, enabling the body to rid itself of the malignancy [27]. Breyanzi was demonstrated to be efficacious in two clinical trials including over 300 adult patients with DLBCL. According to these trials, 53% and 33% of patients treated with Breyanzi had a full reply (no symptoms of malignancy following therapy), while 73% and 61% had at least a partial response [28].

On June 24, 2022, the FDA approved lisocabtagene maraleucel, known as Breyanzi, for use. Also Breyanzi obtained an EU-wide marketing authorisation on April 4, 2022.

Carvykti (Ciltacabtagene autoleucel)

Multiple myeloma is a kind of cancer that affects plasma cells, a type of white blood cell. This is a kind of bone marrow cancer that grows and multiplies fast in the bone marrow. It is a condition characterized by an uncontrollable growth in plasma cells. Myeloma cells are plasma cells that appear in multiple myeloma. Because multiple myeloma only develops in the bone marrow, malignant cells force out healthy bone cells [29].

Ciltacabtagene autoleucel, also known as Carvykti, a CAR-T therapy, is a gene therapy product used to treat multiple myeloma. A gene treatment called Carvykti uses the patient's own T cells that have been genetically altered in a lab to produce a protein known as the CAR. The chimeric antigen receptor binds to a protein called B cell maturation antigen, which is found on the surface of multiple myeloma cells. When Carvykti is administered to the patient, the modified T cells bind to the B cell maturation antigen and after binding kill the myeloma cells, thereby clearing the multiple myeloma from the body. Carvykti was found to be successful in eliminating cancer cells in individuals with multiple myeloma who had not responded to three or more prior therapies in the clinical research. After one and a half years, roughly 84% (95 of 113 patients) of Carvykti patients had a satisfactory response to treatment, and 69% (78 of 113 patients) had no symptoms of malignancy [30,31].

Carvykti was authorized by the FDA on February 28, 2022 for individuals with multiple myeloma that is not responding to therapy or has returned following treatment. On May 25, 2022, ciltacabtagene autoleucel was authorized for medicinal use in the European Union.

Abecma (Idecabtagene vicleucel)

Mulitple myeloma is a type of bone marrow cancer that results from an overgrowth of plasma cells. Plasma cells develop from B-lymphocytes, which are white blood cells. When bacteria or viruses enter the body, some B-lymphocytes turn into plasma cells, and these produce different protein defense substances called antibodies for each type of bacteria or virus. These antibodies fight bacteria or viruses that enter the body. In multiple myeloma, the increased abnormal plasma cells produce an abnormal protein. These abnormal proteins cannot fight infections and even increase in excessive amounts, causing a decrease in blood fluidity, damaging the kidneys [29].

Adults with multiple myeloma can be treated with the cell-based gene therapy drug idecabtagene vicleucel, also marketed as Abecma. Adults with at least three prior treatments—including immunomodulatory drugs, proteasome inhibitors, and anti-CD38 antibodies—and whose condition has gotten worse since the last therapy are the target population for it [32].

Abecma comprises the patient's own T cells that have been genetically altered in a lab to produce a protein known as CAR. A protein on the surface of cancer cells known as B cell maturation antigen (BCMA) is a target for CAR. When the patient receives this gene therapy, the changed T cells connect to the BCMA and begin destroying cancer cells, cleansing the body of malignant cells. Abecma proved successful in curing the malignancy in one major study of 140 individuals with multiple myeloma without resistant myeloma and with recurrent myeloma. After receiving Abecma medication, 30% of patients experienced a full response, meaning no evidence of cancer was present, and 67% of patients saw at least a partial response [33].

In March 2021, idecabtagene vicleucel received medical approval for use in the US. The FDA has authorized it as the first cell-based gene therapy for multiple myeloma. In August 2021, it received European Union medicinal use approval.

3. Conclusion

In 2022, 8 gene and cell therapy products were approved for use by the EMA and FDA. These products have always been the most expensive drugs. The most expensive gene therapy product up to this year is Zolgensma (Onasemnogene Abeparvovec) for the treatment of SMA, with a price of over 2 million dollars. But with Hemgenix (Etranacogene dezaparvovec) approved this year, that has changed. This gene therapy product is now the most expensive gene therapy product approved for use in the treatment of hemophilia B. The year 2022 has been revolutionary for hemophilia. Two new gene therapy products were applied for both hemophilia A and hemophilia B therapy. Apart from these, gene and cell therapy products have been approved for AADC deficiency, lymphoma and multiple myeloma treatments. One of the problems with gene and cell therapy products is that they are used in the market rather than producing them. Because these products are mostly used to treat rare diseases and the number of

patients is less than other diseases. Manufactured by BlueBirds Bio and approved in Europe, Skysona pulled it off the market just one month after it was approved because they couldn't agree on a price. BlueBirds Bio company pulled Zynteglo (in 2019), which was approved by the EMA 2 years before Skysona (in 2021), from the market for the same reasons. After these, the company stopped all its activities in Europe and aimed to reach the American market [4]. These two gene and cell therapy products, produced by BlueBirds Bio and withdrawn from the European market this year, have been approved for use by the FDA.

References

1) Kaji EH, Leiden JM. Gene and Stem Cell Therapies. JAMA. 2001;285(5):545–550. doi:10.1001/jama.285.5.545

2) Center for Biologics Evaluation and Research. (n.d.). *What is gene therapy?* U.S. Food and Drug Administration. Retrieved December 13, 2022, from https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/what-gene-therapy

3) Center for Biologics Evaluation and Research. (n.d.). Approved cellular and gene therapy products. U.S. Food and Drug Administration. Retrieved December 13, 2022, from https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products

4) Yıldırım, S., & Kocabaş, F. (2021). Gene therapy products reached to market by 2021. Gene Editing, 2(2), 1–21. https://doi.org/10.29228/genediting.54101

5) Cristina Santoro, Gabriele Quinta Valle, Erminia Baldacci, Antonietta Ferrett, Federica Riccardi: Inhibitors in Hemophilia B, Semin Thromb Hemost 2018; 44(06): 578-589

6) Biggs R, Douglas AS, Macfarlane RG, Dacie JV, Pitney WR, Merskey C. Christmas disease – a condition previously mistaken for haemophilia. Br Med J 1952; 2: 1378–82.

7) Goodeve, A. C. (2015). Hemophilia b: Molecular Pathogenesis and mutation analysis. Journal of Thrombosis and Haemostasis, 13(7), 1184–1195. https://doi.org/10.1111/jth.12958

8) Annette Von Drygalski, Adam Giermasz, Giancarlo Castaman, Nigel S. Key, Susan Lattimore, Frank W. G. Leebeek, Wolfgang Miesbach, Michael Recht Alison Long, Robert Gut, Eileen K. Sawyer, Steven W. Pipe, Etranacogene dezaparvovec (AMT-061 phase 2b): normal/near normal fix activity and bleed cessation in hemophilia B, OCTOBER 30, 2019.

9) Courtney D. Thornburg, Etranacogene dezaparvovec for hemophilia B gene therapy, First published online on November 24, 2021.

10) Mr. Sachin Navale, Miss. Kaveri Bhosale, Mr. Mahesh Mohite, & Mrs. Swapnali Navale. (2022). Hemgenix as first gene therapy for treatment of haemophilia B. International Journal of Advanced Research in Science, Communication and Technology, 89–94. https://doi.org/10.48175/ijarsct-7657

11) Center for Biologics Evaluation and Research. (n.d.). Hemgenix. U.S. Food and Drug Administration. Retrieved December 8, 2022, from https://www.fda.gov/vaccines-blood-biologics/vaccines/hemgenix

12) Naddaf, M. (2022, December 6). Researchers welcome $3.5-million haemophilia gene therapy - but questions remain. Nature News. Retrieved December 8, 2022, from https://www.nature.com/articles/d41586-022-04327-7

13) Mannucci PM, Tuddenham EGD. The hemophiliac – from royal genes to gene therapy. N Engl J Med. 2001;344:1773–1779. doi: 10.1056/NEJM200106073442307.

14) Sheridan, C. For hemophilia and thalassemia, a new era of 'one-and-done' gene therapies has arrived. Nat Biotechnol 40, 1531–1533 (2022). https://doi.org/10.1038/s41587-022-01555-0

15) Ema. (2022, November 9). Roctavian. European Medicines Agency. Retrieved December 8, 2022, from https://www.ema.europa.eu/en/medicines/human/EPAR/roctavian-0

16) ICER publishes final report and policy recommendations for hemophilia a therapies. ICER. (2022, January 11). Retrieved December 8, 2022, from https://icer.org/news-insights/press-releases/icer-publishes-final-report-and-policy-recommendations-for-hemophilia-a-therapies/

17) Roctavian: Withdrawn Application | European Medicines Agency. (n.d.). Retrieved December 8, 2022, from https://www.ema.europa.eu/en/medicines/human/withdrawn-applications/roctavian

18) Himmelreich N, Montioli R, Bertoldi M, et al. Aromatic amino acid decarboxylase defciency: molecular and metabolic basis and therapeutic outlook. Mol Genet Metab. 2019;127(1):12–22.

19) Hwu PW, Kiening K, Anselm I, et al. Gene therapy in the putamen for curing AADC defciency and Parkinson's disease. EMBO Mol Med. 2021;13(9): e14712.

20) Ema. (2022, October 24). Upstaza. European Medicines Agency. Retrieved December 8, 2022, from https://www.ema.europa.eu/en/medicines/human/EPAR/upstaza

21) European Medicines Agency. (n.d.). Retrieved December 8, 2022, from https://www.ema.europa.eu/en/documents/assessment-report/upstaza-epar-public-assessment-report_en.pdf

22) Chien YH, Lee NC, Tseng SH, et al. Efcacy and safety of AAV2 gene therapy in children with aromatic L-amino acid decarboxylase defciency: an open-label, phase 1/2 trial. Lancet Child Adolesc Health. 2017;1(4):265–73

23) Keam, S. J. (2022). Eladocagene Exuparvovec: First approval. Drugs. https://doi.org/10.1007/s40265-022-01775-3

24) Lichtman MA. The Non-Hodgkin Lymphomas. Williams Hematology, 7th ed. 2005;1407– 1459. New York: McGraw-Hill.

25) Armitage JO, Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. J Clin Oncol. 1998;16:2780-2795.

26) Annex I summary of Product Characteristics - European Medicines Agency. (n.d.). Retrieved December 9, 2022, from https://www.ema.europa.eu/en/documents/product-information/breyanzi-epar-product-information_en.pdf
27) Ema. (2022, May 20). Breyanzi. European Medicines Agency. Retrieved December 9, 2022, from https://www.ema.europa.eu/en/medicines/human/EPAR/breyanzi
28) Ema. (2020, November 3). European public assessment reports: Background and context - medicines agency. European Medicines Agency. Retrieved December 9, 2022, from https://www.ema.europa.eu/en/medicines/what-we-publish-when/european-public-assessment-reports-background-context
29) Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC. Multiple myeloma. Lancet. 2009 Jul 25;374(9686):324-39. doi: 10.1016/S0140-6736(09)60221-X. Epub 2009 Jun 21. PMID: 19541364.
30) Ema. (2022, September 28). Carvykti. European Medicines Agency. Retrieved December 9, 2022, from https://www.ema.europa.eu/en/medicines/human/EPAR/carvykti
31) Center for Biologics Evaluation and Research. (n.d.). Carvykti. U.S. Food and Drug Administration. Retrieved December 9, 2022, from https://www.fda.gov/vaccines-blood-biologics/carvykti
32) Center for Biologics Evaluation and Research. (n.d.). Abecma. U.S. Food and Drug Administration. Retrieved December 12, 2022, from https://www.fda.gov/vaccines-blood-biologics/abecma-idecabtagene-vicleucel
33) Ema. (2022, August 24). *Abecma*. European Medicines Agency. Retrieved December 12, 2022, from https://www.ema.europa.eu/en/medicines/human/EPAR/abecma

Gene Editing Journal

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Review Article

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(2022) 03: Pages 14-18.
DOI: 10.29228/genediting.66981

The CRISPR-Cas12 SHERLOCK System Can Identify HIV

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Received: 11.12.2022 **Accepted/Published Online**: 21.12.2022 **Final Version**: 25.12.2022

Abstract: The HIV (Human Immunodeficiency Virus) virus can settle in a variety of human tissues and is spread by blood and unprotected sexual contact, but it mostly affects the immune system. Today's anti-HIV medications stop the virus's growth in the body and its immunosuppressive effects, enabling HIV-positive persons to live a longer, healthier life. Early treatment initiation and consistent treatment maintenance under a doctor's supervision are crucial for this. In the treatment and progression of HIV infection, early diagnosis and, consequently, early treatment are important. In addition to increasing life expectancy, early diagnosis also lowers transmission rates. Today, RT-PCR and antibody-based diagnostic kits are used for HIV diagnosis. But both diagnostic models have some flaws and glitches. To eliminate this situation, CRISPR-based diagnostic kits are very suitable for use in the diagnosis of both viral and bacterial diseases.

Key words: Human Immunodeficiency Virus, Sexual Transmission Disease, Diagnostic kits, CRISPR Cas-12a, SHERLOCK System.

1. Introduction

HIV is a virus that directly damages the immune system, and people who carry this virus are called “HIV positive”. HIV reduces body resistance, causing people to get sick easily. AIDS (Acquired Immune Deficiency Syndrome) is a disease that occurs after HIV weakens the immune system. The United Nations HIV/AIDS Joint Program (UNAIDS) 2019 report states that since the start of the global HIV epidemic, 74.9 million individuals have contracted the virus and 32 million have passed away from AIDS-related illnesses. There are 22,345 HIV(+) people and 1864 AIDS cases in our country, from 1985 when the first case was seen, until 10 November 2019, when the confirmatory test was positive and reported. A person who has a suspicious relationship or has a test can be done in other health institutions after 21 days. Due to the Covid-19 pandemic process, people have avoided hospitals and such health institutions. At the same time, it may be the case when the person voluntarily and voluntarily avoids having a test in a health institution. Considering these two situations; The individual needs to do his/her test easily in his/her private area, at home, and to learn the result quickly. Nucleic acid detection is an essential approach for disease identification, but may be costly, have inconsistent sensitivity and specificity, and need extensive infrastructure. The essential need for transformational infectious disease detection that can be used in a wide range of clinical situations and against a variety of pathogens is the driving force behind the enthusiasm around the development of CRISPR/Cas-based diagnostics. Such testing has the potential to be applied to both clinical care and surveillance for pathogen outbreaks.

The term "Sexually Transmitted Diseases" refers to conditions that spread from one person to another through sexual interaction. Additionally known as sexually transmitted infections, this group of illnesses. Blood, semen, vaginal fluid, and other bodily fluids can be used as transmission vehicles for the bacteria, viruses, or fungi that cause STDs. STD can spread between people without sexual contact. For example, these infections can be passed from mother to baby during pregnancy or birth. Conditions such as blood transfusion (transfusion) and the use of joint needles, especially in drug users, are among the ways of spreading sexually transmitted diseases.

2. Virus

Viruses are ellipse, round, non-uniform bar, hexagonal, etc. are found in shapes. Viruses do not show cell structure. Their structure consists of a nucleic acid core and capsid (protein sheath) surrounding it. Some viruses have a viral membrane, or envelope, surrounding the viral nucleocapsid. For this reason, 3 basic structural features are seen in viruses; Capsomere and capsid, Envelopes and Nucleic acids. The viral genome is the only DNA or RNA present in the nucleic acids that make up the

genetic architecture of viruses. The genetic material needed to create nucleic acid is stored inside the viral genome and delivered to the host cell.

Viruses, which means poison in Latin, are factors that cause infectious diseases in humans, animals and plants, and are important both economically and medically. Unlike bacteria, viruses are obligate intracellular parasites. Except for living cells; they cannot replicate, interact, or lose their potential to infect to act in environmental conditions. Viruses need a living cell to reproduce, so they are not active outside of the living cell.

3. Human Immunodeficiency Virus (HIV) I-II

HIV-1 is the etiological agent of AIDS, which emerged in the 1980s. HIV, which is thought to have been transmitted to humans from ape species zoonotically, is a retrovirus and is classified in the genus Lentivirus. In 1986, HIV-2, which is related to HIV-1 but immunologically different, was identified. Pandemic HIV-1 has been reported in all countries in the world, while HIV-2 has been limited to western African countries. HIV-1 isolates were grouped into four genetic groups, M (main/ major), O (outlier), N (non-M, non-O) and P, according to sequence differences in gag and env genes. HIV-1 group M consists of 9 subgroups and constitutes 98% of the strains isolated all over the world. HIV is an enveloped, positive-sense RNA virus. Mature viral particles are 100-150 nm in diameter and contain a conical core surrounded by a lipid envelope. The chord contains two identical RNAs of approximately 10 kb in length. The genome contains three gene regions, gag, pol and env, which encode structural proteins, viral enzymes and envelope glycoproteins, respectively. HIV binds to CD4 molecules on the surface of susceptible cells by the envelope protein gp120/41 originating from the gp160 precursor encoded by the env gene. However, the entry of HIV-1 into the cell also requires its binding to the core-receptors CXCR4 and/or CCR5.
CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats)

Its working principle is based on RNA-mediated nucleases (Bortesi et al. 2015). The most widely used system is the CRISPR/Cas9 system discovered in Streptococcus pyogenes (Rani et al. 2016; Nejat et al. 2016). The CRISPR system is part of the immune systems of bacteria and archaea to protect their genomes. This system cuts foreign DNA depending on its sequence, protecting bacteria and archaea from invading nucleic acids (such as viruses) (Jinek et al. 2012; Hwang et al. 2013).

The Cas9 protein is guided by the two CRISPR RNAs crRNA and tracrRNA in the original CRISPR system found in bacteria. A single RNA known as sgRNA (single guide RNA), which is created by joining the 3' region of the crRNA and the 5' end of the tracrRNA, is present in the reprogrammed CRISPR system utilized in genome editing (Bortesi et al., 2015). In this approach, the DNA endonuclease Cas9 and a 20 nucleotide RNA sequence created in accordance with the region of the genome that will be targeted are all that is required to take advantage of CRISPR technology. Double-strand breaks happen in the target area of the DNA when these two system components connect to it and create a protein (Cas 9) - RNA (SgRNA) - DNA (genomic DNA) complex (Fichtner et al. 2014; Schaeffer et al. 2015). The presence of an NGG sequence called PAM at the 3' end of the target region is the only need for the identification of the target region and for the Cas9/sgRNA complex to slice on DNA (Gasiunas et al. 2012; Mahfouz et al. 2014).

With the demonstration in early 2013 that the Cas9 system could be used for genome modification of human cell cultures, it was understood that the CRISPR system could be used in other eukaryotic organisms (Schiml et al. 2016). The biggest issue with studies on genome editing is the off-target effect. This is because genome editing tools might cut and produce other undesirable alterations even though they are intended to target a particular section of the genome. The presence of another sequence or sequences in the genome that is identical to the targeted sequence frequently causes this miscut.

If the complete genome sequence of the organism studied is known, the presence and frequency of possible non-specific sequences in the genome can be determined by BLAST application (Jia et al. 2014; Nejat et al. 2016). To determine this frequency, different algorithms have been developed by scientists and web-based computer programs have been created. Two of the most widely used programs for CRISPR are CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) and CRISPR-DESIGN (http://crispr.mit.edu/).

Some reports report off-target effects and question the specificity of the CRISPR system, as well as studies in which no off-target effects have been observed (Ceasar et al. 2016; Pan et al. 2016). The resulting off-target activity varies from organism to organism (Pan et al. 2016). Many different ways have been proposed to reduce off-target activity arising from the working mechanism of the CRISPR system. Using sgRNA short of 20 nucleotides and using dual Cas9 nickases, each of which creates a single strand cut, can be given as examples of these ways (Lawrenson et al. 2015; Paul et al. 2016; Rani et al. 2016). The synchronous silencing of homologous genes with a single sgRNA is one of the CRISPR system's key benefits (Endo et al. 2015; Zhang et al. 2016). On the other hand, by using Cas9/sgRNA expression vectors containing multiple sgRNAs, the functions of members of a gene family in their pathways can be examined (Zhang et al. 2016).

Studies have shown that thanks to the ability of Cas9 and sgRNA to be sent into the cell as protein and RNA, gene transfer between organisms, which is one of the main causes of concern in GMOs, and the possible problems that this may bring can be prevented (Woo et al. 2015). Work continues on the optimization of CRISPR technology for more precise and effective genome editing. Numerous applications in human health and biotechnology, such as the identification of infectious diseases, agricultural pathogens, or circulating DNA or RNA linked to disease, depend heavily on rapid nucleic acid detection (Sapkota ve ark. 2020). Although successful, conventional techniques for amplifying nucleic acids for detection (such as PCR) need instrumentation that is not portable, preventing their use in the field (Eldin ve ark. 2019). Approaches based on CRISPR-Cas are being studied for the treatment of infectious, genetic, and many other disorders. There have been several CRISPR-Cas- based methods created to date for the detection and diagnosis of infectious and non-infectious disorders. CRISPR-based technologies are becoming more prevalent in the field of molecular diagnostics and could soon displace PCR in several applications (Uppada et al., 2018).

CRISPR-Cas systems were created for the first time in 2016 to recognize nucleic acids for molecular diagnostics. The development of numerous isothermal amplification techniques has been prompted by the demand for instrument-free nucleic acid detection technologies (Yuan ve ark. 2019). Recombinase polymerase amplification (RPA), a popular method for isothermal amplification, however, calls for optimization and frequently is unable to distinguish between single-base-pair changes in target DNA sequence, a distinction that can have significant pathogenicity implications (Macdonald et al., 2018). CRISPR-Cas system enzymes have recently been modified for the precise, quick, sensitive, and portable detection of nucleic acids. Recently, a wide variety of CRISPR-based techniques for identifying nucleic acids were described. Early technologies used either the modified nucleolytically null Cas9 (dCas9) protein or the canonical Cas9 protein of type II CRISPR-Cas systems (Wang et al., 2016). The identification of Cas12 and Cas13's protein collateral activity is a significant step toward the development of CRISPR-based molecular diagnostics. The CRISPR systems' Cas13 and Cas12 protein families, both of which have so far been demonstrated to exhibit collateral action, are helpful in applications involving nucleic acid identification.

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SHERLOCK, a CRISPR-Cas type VI system-based diagnostic tool, was introduced in 2018 by Zhang and his team. Similar in concept to DETECTR, SHERLOCK depends on the action of the Cas13 nuclease from Leptotrichia wadei. Instead of recognizing and cleaving DNA as Cas12a does, Cas13 detects and cleaves only RNA, which nonspecifically corrupts any nearby RNA molecules. Recognition of DNA targets is made possible by in vitro transcription of the isolate. Increasing sensitivity and enriching target molecules are both possible with isothermal amplification RPA. The Cas13 protein crRNA and fluorescent RNA probes are combined with the amplified RNA fragments. The connection between the fluorophore and the quencher is broken down if the target molecules are present in the sample because Cas13 detects them via crRNA and indiscriminately cleaves (via collateral activity) fluorescent RNA probes. Thus, the presence and strength of the fluorescent signal show how much of the target is present in the biological sample. The scientists showed that SHERLOCK has attomolar sensitivity for the detection of the Zika virus, dengue virus, different harmful microorganisms, and SNPs in DNA. The SHERLOCK's fundamental flaw was that it was entirely qualitative and not quantitative, but a year later, the authors unveiled the SHERLOCKv2 as a replacement (Gootenberg et al., 2018).

It can be helpful to screen for several sequences at once in a clinical or field environment to distinguish between viruses that cause comparable symptoms. To do this, Zhang Group also included a multiplex option in SHERLOCKv2. The discovery that the nonspecific trans-cleavage activities of Cas13 from various species had substantially skewed and distinct preferences for particular sequence motifs allowed for multiplexing. For instance, PsmCas13B from Prevotella sp. MA2016 prefers rG-rA dinucleotides over rA-rU dinucleotides, whereas LwaCas13a from Lachnospiraceae bacterium NK4A179 has the reverse

preference. As a result, the activity of the corresponding enzymes can be distinguished using reporter probes labelled with various fluorophores and each containing a unique nonspecific cleavage motif. The corresponding enzymes also have orthogonal guide RNA sequences that can distinguish the multiplex target sites (Myhrvold et al., 2018).

4. Discussion

The field of viral infection is the one where CRISPR-based diagnostic methods have received the greatest attention. Tools named DETECTR and SHERLOCK which are based on the CRISPR-Cas12a and Cas13a families have been developed by several scientists. DETECTR uses the Type V Cas12a enzyme to directly connect to DNA targets by first directing the Cas12a enzyme to a double-stranded DNA sequence within a specific viral DNA. After the genome connects to its viral genetic target, the enzyme Cas12a indiscriminately cleaves a single-stranded DNA molecule connected to a quencher molecule and a reporter fluorophore (Kocak et al. 2018). This "collateral" cleavage is recognized by the release of a fluorescent signal from the fluorophore and quencher. The primary advantage of the DETECTR approach is its high sensitivity, which makes it possible to detect a single viral particle molecule in a microliter sample (Kocak et al. 2018). The Type VI Cas13a enzyme is used in the SHERLOCK technique to indiscriminately bind and cleave RNA to crRNA targets (Gootenberg et al. 2018). The target RNA is bound by a targeting molecule with an attached fluorophore, which causes it to be cut sideways. When specific sequences are present, this generates a fluorescence signal, which can be used to identify and confirm the presence of viral DNA. SHERLOCK's uses in the identification and diagnosis of viruses have been the subject of in-depth research. Since then, scientists have further enhanced the method by developing the SHERLOCKv2 protocol, which is succinct and more targeted. The use of multiplexing enables the fluorescence reporting of the identification of four different RNA target sequences in a single experiment. Multiplexing is accomplished by identifying orthogonal sequencing capabilities by optimizing Cas13a and Cas13b family enzymes. The Cas13 enzymes were combined with the Csm6 enzyme associated with CRISPR, which more than tripled sensitivity. The DETECTR and SHERLOCK technologies can be used to detect a range of viruses in both clinical and laboratory settings. Although the DETECTR method has mostly been used to identify the human papillomavirus, it can theoretically be used to identify any virus (HPV). The SHERLOCK and DETECTR approaches can be combined with recombinase polymerase amplification (RPA) to improve viral material amplification and detection. The SHERLOCK method can also be enhanced for the detection of HIV, which is still a major viral infection on a global scale. (Bhattacharyya et al. 2018)

References

Jolles, S., Orange, J. S., Gardulf, A., Stein, M. R., Shapiro, R., Borte, M., & Berger, M. (2015). Current treatment options with immunoglobulin G for the individualization of care in patients with primary immunodeficiency disease. Clinical and experimental immunology, 179(2), 146–160. https://doi.org/10.1111/cei.12485

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N. J., & Nekrasov, V. (2015). Editing plant genomes with CRISPR/Cas9. Current opinion in biotechnology, 32, 76–84. https://doi.org/10.1016/j.copbio.2014.11.007

Tang, X., Zheng, X., Qi, Y., Zhang, D., Cheng, Y., Tang, A., Voytas, D. F., & Zhang, Y. (2016). A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants. Molecular plant, 9(7), 1088–1091. https://doi.org/10.1016/j.molp.2016.05.001

Voytas D. F. (2013). Plant genome engineering with sequence-specific nucleases. Annual review of plant biology, 64, 327–350. https://doi.org/10.1146/annurev-arplant-042811-105552

Harnessing CRISPR Effectors for Infectious Disease Diagnostics Roby P. Bhattacharyya, Sri Gowtham Thakku, Deborah T. (2018) Hung10.1021/acsinfecdis.8b00170ACS Infectious Diseases

All-in-One Dual CRISPR-Cas12a (AIOD-CRISPR) Assay: A Case for Rapid, Ultrasensitive and Visual Detection of Novel Coronavirus SARS-CoV-2 and HIV virus 2020Xiong Ding, Kun Yin, Ziyue Li, Changchun Liu10.1101/2020.03.19.998724 CRISPR-Based Diagnosis of Infectious and Noninfectious Diseases

Somayeh Jolany vangah, Camellia Katalani, Hannah A. Boone, Abbas Hajizade, Adna Sijercic, Gholamreza Ahmadian (2020). 10.1186/s12575-020-00135-3Biological Procedures Online SHERLOCK: nucleic acid detection with CRISPR nucleases

Max J. Kellner, Jeremy G. Koob, Jonathan S. Gootenberg, Omar O. Abudayyeh, Feng Zhang. (2019). 10.1038/s41596-019-0210-2Nature Protocol. 52

Norimitsu Hosaka, Nicaise Ndembi, Azumi Ishizaki, Seiji Kageyama, Kei Numazaki, Hiroshi Ichimura (2009). Rapid detection of human immunodeficiency virus type 1 group M by a reverse transcription-loop-mediated isothermal amplification assay. 10.1016/j.jviromet.2009.01.004Journal of Virological Methods

Shen, H., Strunks, G.D., Klemann, B.J., Hooykaas, P.J., & de Pater, S. (2016). CRISPR/Cas9-Induced Double-Strand Break Repair in Arabidopsis Nonhomologous End-Joining Mutants. G3: Genes|Genomes|Genetics, 7, 193 - 202.

Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., & Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. The EMBO journal, 17(18), 5497–5508. https://doi.org/10.1093/emboj/17.18.5497

Sonoda, E., Hochegger, H., Saberi, A., Taniguchi, Y., & Takeda, S. (2006). Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. DNA repair, 5(9-10), 1021–1029. https://doi.org/10.1016/j.dnarep.2006.05.022

Szczepek, M., Brondani, V., Bu¨chel, J., Serrano, L., Segal, D. J., & Cathomen, T. (2007). Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. Nature biotechnology, 25(7), 786–793. https://doi.org/10.1038/nbt1317

Osakabe A, Tachiwana H, Kagawa W, Horikoshi N, Matsumoto S, Hasegawa M, Matsumoto N, Toga T, Yamamoto J, Hanaoka F, Thoma¨ NH, Sugasawa K, Iwai S, Kurumizaka H. Structural basis of pyrimidine-pyrimidone (6-4) photoproduct recognition by UV-DDB in the nucleosome. Sci Rep. 2015 Nov 17;5:16330. doi: 10.1038/srep16330. PMID: 26573481; PMCID: PMC4648065.

Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., & Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. Nature reviews. Genetics, 11(9), 636–646. https://doi.org/10.1038/nrg2842

Petolino J. F. (2015). Genome editing in plants via designed zinc finger nucleases. In vitro cellular & developmental biology. Plant : journal of the Tissue Culture Association, 51(1), 1–8. https://doi.org/10.1007/s11627-015-9663-3

Budhagatapalli, N., Rutten, T., Gurushidze, M., Kumlehn, J., & Hensel, G. (2015). Targeted Modification of Gene Function Exploiting Homology-Directed Repair of TALEN-Mediated Double-Strand Breaks in Barley. G3 (Bethesda, Md.), 5(9), 1857–1863. https://doi.org/10.1534/g3.115.018762

Moscou, M. J., & Bogdanove, A. J. (2009). A simple cipher governs DNA recognition by TAL effectors. Science (New York, N.Y.), 326(5959), 1501. https://doi.org/10.1126/science.1178817

Guilinger JP, Pattanayak V, Reyon D, Tsai SQ, Sander JD, Joung JK, Liu DR. Broad specificity profiling of TALENs results in engineered nucleases with improved DNA- cleavage specificity. Nat Methods. 2014 Apr;11(4):429-35. doi: 10.1038/nmeth.2845. Epub 2014 Feb 16. PMID: 24531420; PMCID: PMC4010127.

Reyon, D., Tsai, S. Q., Khayter, C., Foden, J. A., Sander, J. D., & Joung, J. K. (2012). FLASH assembly of TALENs for high-throughput genome editing. Nature biotechnology, 30(5), 460–465. https://doi.org/10.1038/nbt.2170

Bortesi, L., & Fischer, R. (2015). The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnology advances, 33(1), 41–52. https://doi.org/10.1016/j.biotechadv.2014.12.006

Magdy M. Mahfouz, Agnieszka Piatek, Charles Neal Stewart (2014). Genome engineering via TALENs and CRISPR/Cas9 systems: challenges and perspectives 10.1111/pbi.12256Plant Biotechnology Journal

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Review Article

©Gene Editing
(2022) 03: Pages 19-29.
DOI: 10.29228/genediting.66978

Development of DNA Ink & DNA Barcoding Technology on Practical Tracking & Authenticity Detection of Pharmaceutical Ingredients

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Received: 11.12.2022 **Accepted/Published Online**: 21.12.2022 **Final Version**: 31.12.2022

Abstract: Every year, millions of drugs are thrown away by consumers, even before their expiration date, assuming they have deteriorated. We need to see the fact that this has negative repercussions for our country and the world. In addition, when the data of the World Health Organization is examined, when it is considered that approximately 50% of the drugs are used incorrectly or unnecessarily or they are thrown away without even opening the box, we can see that the waste is large. We should not forget that the waste of drugs also brings environmental problems. It is crucial to maintain proper medicine storage conditions and keep out anything that could cause them to deteriorate. The most important factors that play a role in the deterioration of drugs are light, temperature and humidity. If drugs are not stored under appropriate conditions, their chemical structures may change and their effectiveness may decrease or disappear. One of the main factors affecting the deterioration of drugs is that drugs are not kept in the cold chain except for heat, light and humidity. These factors affecting degradation also affect the degradation of DNA. Therefore, it is anticipated that a DNA-based ink will be developed and used in the packaging of drugs, and drug degradation can be followed easily. As a groundbreaking project in the field of health, it is aimed that the person using the drug will be able to notice the deterioration in drugs over time with the DNAINK method. With DNAINK, it is aimed to understand whether the products still maintain their stability effectively, since the text on the drug boxes will be written with DNA ink. The DNA will be fluorescently marked, placed in ink, and injected into the writing on the medicine box. The target is that if the double-stranded DNA is left in conditions such as ultraviolet, light, temperature and humidity for a long time or if it is dependent on time, breaks will occur in the ink containing DNA, and it is aimed to determine the rate of degradation by following these breaks. Against counterfeit drug production, which has become a major health threat in the market, the DNAINK project will additionally develop barcoding technology, an important solution to this problem. Aims to bring Barcoding on the purchased medicine box will help to understand that the medicine came out of the right production place, and to bring a great solution to the health problems experienced due to counterfeit medicine production. With the barcoding technology, it will be possible to monitor whether the purchased drug is fake or not and whether the material deteriorates over time with the DNAINK method. Restriction enzyme analysis will be performed in plasmid DNA design and pHIV-EGFP plasmid will be used at this stage. Then the DNA will be mixed with the ink, and this ink will be subjected to certain tests to measure its stability and effectiveness. Afterwards, it is aimed to reach the desired stage of the project by performing DNA barcode sequencing and analysis. Within the scope of the realization of this project, it is aimed to make it easier to follow the deterioration of the drugs used due to any factor before the expiry date, to protect human health against counterfeit drug production and to guarantee the accuracy of the production site of the used drug.

Key words: DNA Ink, Medicine Chest, DNA, Counterfeit Drug

1. Introduction

Every year, millions of drugs are thrown away by consumers, even before their expiration date, assuming they have deteriorated. We need to see the fact that this has negative repercussions for our country and the world. In addition, when the data of the World Health Organization is examined, when it is considered that approximately 50% of the drugs are used incorrectly or unnecessarily or they are thrown away without even opening the box, we can see that the waste is large. We should not forget that the waste of drugs also brings environmental problems. It is crucial to maintain proper medicine storage conditions and keep out anything that could cause them to deteriorate. The most important factors that play a role in the deterioration of drugs are light, temperature and humidity. If drugs are not stored under appropriate conditions, their chemical structures may change and their effectiveness may decrease or disappear. One of the main factors affecting the deterioration of drugs is that drugs are not kept in the cold chain except for heat, light and humidity. These factors affecting degradation also affect the degradation of DNA. Therefore, it is anticipated that a DNA-based ink will be developed and used in the packaging of drugs, and drug degradation can be followed

easily. As a groundbreaking project in the field of health, it is aimed that the person using the drug will be able to notice the deterioration in drugs over time with the DNAINK method. With DNAINK, it is aimed to understand whether the products still maintain their stability effectively, since the text on the drug boxes will be written with DNA ink. The DNA will be fluorescently marked, placed in ink, and injected into the writing on the medicine box. The target is that if the double-stranded DNA is left in conditions such as ultraviolet, light, temperature and humidity for a long time or if it is dependent on time, breaks will occur in the ink containing DNA, and it is aimed to determine the rate of degradation by following these breaks. Against counterfeit drug production, which has become a major health threat in the market, the DNAINK project will additionally develop barcoding technology, an important solution to this problem. Aims to bring Barcoding on the purchased medicine box will help to understand that the medicine came out of the right production place, and to bring a great solution to the health problems experienced due to counterfeit medicine production. With the barcoding technology, it will be possible to monitor whether the purchased drug is fake or not and whether the material deteriorates over time with the DNAINK method. Restriction enzyme analysis will be performed in plasmid DNA design and pHIV-EGFP plasmid will be used at this stage. Then the DNA will be mixed with the ink, and this ink will be subjected to certain tests to measure its stability and effectiveness. Afterwards, it is aimed to reach the desired stage of the project by performing DNA barcode sequencing and analysis. Within the scope of the realization of this project, it is aimed to make it easier to follow the deterioration of the drugs used due to any factor before the expiry date, to protect human health against counterfeit drug production and to guarantee the accuracy of the production site of the used drug. With the DNA-based ink used in the packaging of drugs, it is aimed to detect the deterioration of drugs easily, to track and monitor the deterioration of drugs in case they lose the appropriate environmental conditions, and to ensure the originality of the drug with barcoding technology.

2. Medicines in the Health Sector

The pharmaceutical industry is an important industry that plays a role in protecting the health and improving the quality of life, which is the most basic need of human beings. The pharmaceutical industry producing for health today has a dynamic structure which has the effect of extending the life span of people, increasing life expectancy due to increasing income levels, factors environment and technological development. The fact that the drug cannot be replaced, that is, other products cannot replace the drug, distinguishes the drug from other cheap products. Due to this property, the pharmaceutical industry is the most critical today. Due to the importance, they attach to the pharmaceutical industry, most countries are involved in drug pricing and can intervene in the import and export of drugs and drugs used in drug manufacturing. In addition to its importance for economic development, it is essential to find a pharmaceutical industry capable of producing to meet the country's pharmaceutical needs in cases such as epidemics, possible embargoes and wars. This situation has included the COVID-19 epidemic, which spread rapidly around the world and caused the deaths of hundreds of thousands of people. So much so that during the epidemic there have been cases in which countries have seized medicines and materials purchased from third countries, because countries did not have sufficient medicines and protective equipment and did not produce for the treatment of their citizens. Therefore, the pharmaceutical industry is a strategic industry that countries need to establish and develop. Today, pharmaceuticals have become an indispensable part of healthcare systems around the world. Historically, pharmaceuticals have played a crucial role in human development, improving quality of life and reducing time spent in hospitals. Thanks to the innovative pharmaceutical industry, almost all epidemics and chronic diseases can be cured today. Due to its direct link to the welfare and well-being of the people, the pharmaceutical industry is of strategic importance for the development of a healthy and productive nation. Today, the pharmaceutical industry is recognized as one of the largest and fastest-growing global industries. It is an important source of job creation and foreign exchange earnings for many countries around the world. However, despite all these extraordinary results, it is a harsh reality that millions of people around the world die every year, mainly in low-income developing countries, due to the unavailability and inaccessibility of the necessary drugs. According to the World Health Organization (WHO), an average of 30% of the world's population does not have access to life-saving drugs; while in some countries of Asia and Africa this number can reach 50% (Roger Bate, 2008). Many developing countries, including some OIC Member States, have little or no production capacity in the pharmaceutical industry. The local industry covers a small part of the domestic demand for medicines and is heavily dependent on imports and medical assistance. Furthermore, the share of pharmaceuticals in "out-of-pocket" (ie, paid for by the patient) healthcare payments in these countries varies between 40 and 60%. As a result, medicines are not available or accessible to a large part of the population, and hundreds and thousands of people die from preventable and treatable diseases.

3. Pharmaceutical Expenditures in the Health Sector

Today, the pharmaceutical industry is gaining attention as an important part of the healthcare sector. Over the past decade, the pharmaceutical industry has played and continues to play an important role in reducing mortality and morbidity. State interventions in the pharmaceutical industry concern both health policy and economic policy. According to data from the

Association of Pharmaceutical Industry Employers of our country in 2010, the Turkish prescription drug market decreased by 1.2% on the volume scale, reaching 13.9 billion TL (9 .2 billion USD) and up 2.8% on the unit scale. Reach 1.45 billion boxes. Drug consumption per capita was $133 in 2010. In 2010, 1.45 billion boxes of drugs were put on the market, an increase of 2.8% compared to 2009. While the number of drugs consumed was 14.1 billion TL in 2009, the number of drugs consumed was found to be 13.9 billion TL in 2010.

Although drugs of many treatment groups are imported into our country, preparations that require very new and advanced technologies are imported, some vaccines, blood factors, some controlled release drugs, insulin and anti-cancer drugs. Medicines and their raw materials are imported into all countries, even the most developed ones. The pharmaceutical industry is behind in terms of its capacity and the foreign trade balance speaks in favour of imports. By supporting the production and use of equivalent drugs, it will be possible to increase Turkey's competitiveness and develop its export potential in this direction(Akar, A., 2004).

According to 2020 TUIK data, the total health expenditure in 2020 increased by 24.3% compared to the previous year and reached 249 billion 932 million TL. Health expenditure by public administrations increased by 26.3% and reached 198 billion and 62 million Turkish lira. Private sector health expenditure was estimated at TL 869 million 51 billion with an increase of 17.3%. The ratio of general health expenditure to total health expenditure was 79.2% in 2020, while private sector health expenditure was 20.8%. Looking at the sub-components of the state and the private sector, in 2020 the social security institution 51.0%, the central government 27.6%, families 16.0%, insurance companies 2.6%, and non-profit organizations serving households and other businesses. 2.1, local authorities have a 0.7% share.

4. Drug Supply Management

To better and more deeply understand the philosophy of SCM in the healthcare sector, it is necessary to first focus on the differences between the sector. Health facilities provide intangible services alongside tangible products. For instance; While healthcare, which is an intangible service in the treatment of a patient, is supported by tangible products such as medicines; The pharmacy also offers tangible products and intangible services. However, SCM in healthcare has unique selling points. First of all, the end product of the supply chain is the "basic service provided", while the end users of the materials provided are the doctors, nurses and other health workers who are the "internal customers". of the system (Schneller, Smeltzer 2006). The supply chain includes the process of bringing together the raw materials, consumables and production resources (labour, natural resources, capital, technology) to be used in production and delivering them to the production points and then to the final customers at the most convenient cost and the best conditions (Timur, 2013). Manufacturers, drug and pharmaceutical manufacturers, who are key members of the healthcare supply chain; manufacturers of medical devices, such as medical and surgical instruments and devices, prostheses, X-ray equipment, and electrotherapy equipment; information system manufacturers and manufacturers of medical and surgical materials such as syringes, surgical blades, blood and sample kits, hospital laboratory products, wound care units and stents placed in a vein (Burns et al., 2002 Özcan, 2013: 285). -286; Tengilimoğlu & Yiğit, 2013: 28-39) Pharmaceutical industry "chemical, herbal, animal and biological substances used as therapeutic, protective, nutritional and diagnostic tools in human and veterinary medicine, according to pharmaceutical technology and according to scientific standards. 'industry that produces a simple or compound pharmaceutical form in certain doses and produces it in series and puts it at the service of drugs and treatments ”(Abacıoğlu 2010: 47). It turns out that the pharmaceutical industry in our country ranks 17th in the world with a volume of 7 billion dollars and a total of 332 companies are active in the sector (Sarsın Kaya 2016: 3). While 71 of these companies are pharmaceutical manufacturers, 12 companies, including 6 nationwide, produce raw materials nationwide (Sarsın Kaya 2016: 9). In addition, according to the "Turkish Pharmaceutical Industry 2015" report prepared by the Employers' Association of the Pharmaceutical Industry (IEIS), the world's top 10 pharmaceutical manufacturers have sales in the Turkish market; Pfizer from the United States, Novartis from Switzerland and Sanofi from France, which are in the top 3, also produce in Turkey (IEİS 2016). Many institutions implement lean supply chain management in healthcare facilities; Various studies are underway on this. For instance; Yorulmaz, Altunkan, Yasemin and Keleş (2012) tried to obtain false reports in their investigations into the pharmaceutical supply chain in Turkey. In this chain, which includes stakeholders such as manufacturers, warehouses, pharmacies, lending centres and reimbursement institutions, erroneous reports to the drug traceability system were investigated between 2011 and 2012 and products with a date expiration date not allowed to be sold were tempted to sell. These practices can lead to consequences that could pose a problem for human health.

Wholesalers operating in the pharmaceutical sector; "They are companies that supply a flow of products between drug manufacturers and independent retailers, hospitals and pharmacies, including pharmacies, pharmacy chains, major markets and other outlets" (Tengimimoğulu, Yiğit 2013: 34). However, in our country, manufacturers or importers generally distribute drugs to pharmaceutical warehouses and pharmaceutical warehouses to retail pharmacies (Sarsın Kaya

2016: 5). A regulation issued last year revised the definition of pharmaceutical warehouses and added "or workplace permitted by the ministry for the purchase of medicines from abroad for personal care" to the definition (Ministry of Health 2015b). The way to retail is open. Pharmaceutical activity in our country is regulated by the "Law on Pharmacists and Pharmacies". Medicines under the "Ordinance on Pharmacists and Pharmacies" issued by the Turkish Medicines and Medical Devices Authority in 2014, "Preparation and presentation of various pharmaceutical types of medicines from medicinal raw materials of natural origin and synthetic used in the diagnosis and treatment of disease and protection against disease; Drug analysis, monitoring of its pharmacological action in terms of continuity, safety, efficacy and cost; It has been defined as the health service that carries out activities related to the standardization and quality assurance of medicines, informing patients of problems related to the use of medicines and reporting problems. In the same ordinance, a pharmacy is opened as a "health facility providing health services under the responsible ownership and direction of a pharmacist, in accordance with law". An analysis of the structure of the pharmaceutical supply chain in Turkey shows that there are four main players in the production and distribution phase of the drug. These players are drug manufacturers, 3PL service providers, pharmaceutical warehouses and pharmacies. About 75% of drugs produced by manufacturers are delivered to patients through 3PL service providers, drugstores and pharmacies (Bog, 2005).

Manufacturers: Manufacturers of the pharmaceutical industry can be classified according to their field of activity: large manufacturers engaged in research and development and production, small companies engaged in research and development, generic drug manufacturers focused on the production of drugs whose patents are expired and other drug manufacturers that provide the compounds and chemicals needed for the drugs (O'Hanlon, 2017). Pharmaceutical manufacturers have higher profit margins than other stakeholders in the supply chain but are at greater risk due to high R&D costs, ever-increasing standards and high inventory levels (Ay, 2018).

3PL Service Providers: Third-party logistics (3PL) is the provision of logistics services that businesses need from a professional logistics company. In this sense, TPL service providers are specialist logistics companies that manage and control logistics activities on behalf of a shipper (Hertz and Alfredsson, 2003). While 3PL service providers provide a professional logistics service to their customers, they also allow them to focus on their core competencies.

Pharmaceutical Warehouse: Pharmaceutical warehouses store pharmaceutical products from drug manufacturers and 3PL service providers and process them based on pharmacy demand and deliver them on order. Thus, pharmaceutical warehouses connect drug manufacturers, 3PLs and pharmacies. Because drugs are expensive products, pharmacies do not want to work with large stocks of drugs. Instead, pharmacies want to keep a small stock of medicines for retail sale, taking into account short-term needs (Karakoç, 2005). At this point, pharma warehouses act as a buffer between pharmacies and manufacturers and 3PLs. Sell pharmaceutical warehouses by offering their customers overstocks, promotions, discounts or different fulfilment options, based on their market volume. Some pharmaceutical warehouses, on the other hand, do tenders from hospitals and supply and deliver their products according to the specifications presented by the hospital. There are about eighty pharmaceutical warehouse companies in our country.

Pharmacies: Pharmacies are the places where the retail sale of the drug takes place. The pharmacist must have adequate equipment in the process of selling drugs that directly affect human health. For patients to get medications quickly, pharmacies need to consistently and accurately perform activities such as inventory levels, sales forecasts, and tracking of patient records. Pharmacies do not want to work with large stocks of drugs because of the cost of capital. Instead, he prefers to keep a small supply of medicine. This situation makes it necessary for pharmacies to supply frequently and in small volumes from pharmaceutical warehouses. That is why the pharmacy needs to be able to meet the demand for drugs quickly through the distribution channel, just as it is for the patient. Pharmacies prefer to work with pharmaceutical warehouses that minimize the need to hold stock, fulfil orders as quickly as possible, offer various payment options and support sales with promotions. It can be said that applications that require time in sales, such as tracking the drug through tracking systems, controlling all movements and tracking the expiration date, are made faster and more reliable thanks to the collaboration with pharmaceutical warehouses. According to 2018 data from the Turkish Statistical Institute, there are a total of 24,406 pharmacies in the provinces and districts. According to these figures, in our country, there is a pharmacy for every 3,500 inhabitants.

5. Cold Chain Pharmaceuticals

In cold chain logistics, each product needs its heat regimes. These temperature regimes are especially in food transport; It is examined in three stages cool, cold and frosty. Each regime aims to maintain the predetermined temperature during the delivery of products at different temperature ranges. These stages also have cost factors with different dimensions for businesses (Ipekci, 2021).

While some of the inputs used in pharmaceutical production need a cold chain, some do not. Again, temperature monitoring is required during the transport of many types of drugs. Since drugs are also considered perishable products, the ambient temperature must be at certain levels not only during transportation but also while they are kept in pharmacies or drug stores. In addition, the movements of drugs in the research phase, which are still under construction, between laboratories are carried out with the cold chain, while the transportation processes are followed more precisely. The COVID-19 outbreak, which emerged in 2019, has once again made it possible to understand the importance of cold chain logistics in the transport of vaccines and drugs that are already produced and under construction (Won and Lee, 2020).

Batch and deliveries are smaller in vaccine cold chain management. In addition, the preferred temperature in vaccine logistics is generally between 2°C and 8°C. The cold chain management of the vaccine starts at the production center and ends at the point where the vaccine will be administered. Basic cold chain equipment of vaccines; a refrigerator to store vaccines, a digital, electronic or mercury thermometer to record the daily temperature reading, cold boxes to transport and store vaccines, and ice packs to keep vaccines cool. It is also used in materials that separate ice packs from vaccines when using cold boxes (Centers for Disease Control and Prevention, 2003). Vaccines are also kept under control by the equipment in which they are placed in the temperature regime. These equipment are transported to the destination by a standard transport vehicle. The duration of the planned transportation should be calculated correctly so that the temperature regime of the vaccine or drug is not disturbed, and the selection of equipment should be carried out according to this period.

Many deadly diseases in the history of humanity are either not seen or rarely seen on the globe today due to the vaccination of people from birth. These vaccines must be taken from the laboratory environment and delivered to the health institution where the vaccine is made while maintaining the temperature regime. Breaking the temperature regime in the chain may cause the vaccine to lose its properties and lose its effect, and cause social health problems to emerge years later (Kartoğlu and Milstien, 2014). Again, drugs and organs that need to be delivered to patients by the cold chain are of great importance for human and public health.

6. Drug Consumption

Turkey has a developed pharmaceutical industry in terms of technology, standards and capacity. This sector is a sector with high added value, which has the opportunity to produce in significant quantities and varieties, and to export products. Pharmaceuticals are exported to 160 countries around the world. According to the data of the Turkish Statistical Institute (TUIK), while general health expenditures were 61,678 million in 2010, this figure increased to 94,750 in 2014 and 104,568 in 2015. This increase can be explained by the fact that the total population, which was 73.7 million in 2010, increased to 77.7 million in 2014 and 78.7 million in 2015 (TÜİK, 2017). In the Health, Pharmaceuticals and Pharmacy Statistics Yearbook - 2016 booklet published by the Turkish Pharmacists Association (TEB), the total pharmaceutical market in Turkey was 14,598 million TL in 2014, while this figure reached 16,887 million TL in 2015. The Turkish Language Association (TDK) defines medicine as “a substance used in various ways to cure or prevent a disease”. The definition of medicine by the World Health Organization (WHO) is "the product used to change or examine physiological systems and pathological (disease-causing) conditions for the benefit of the user" (Istanbul Chamber of Pharmacists, 2017). The application of the right methods in drug use and the conscious use of the drug is called Rational Drug Use (AİK) (Akıcı and Kalaca, 2013, p. 5). Intelligent drug use is the sum of the rules that patients must take care to provide in accordance with medical needs, at the required dose, at sufficient time intervals, and at the lowest cost for themselves and society (Molahaliloğlu et al., 2011). The definition of RUD was made in Nairobi in 1985 by the World Health Organization (WHO). According to this definition, “It is a set of rules that require patients to take the drugs in accordance with their clinical needs, in doses that meet their personal needs, in a sufficient time frame, at the lowest cost to themselves and the society” (Holloway and Dijk, 2011, p. 1). Misuse and unnecessary use of drugs, ineffective and high-cost drugs cause many problems for the whole world. The use of drugs that cause problems in this way is called Irrational Drug Use (AOIK) (WHO, 2002, p. 5). Smart drug use is a process. This process begins with the patient's identification of their complaints. The AIK process is completed by determining the type of treatment that will eliminate the complaints, selecting and starting the most effective treatment method, prescribing the drugs suitable for the treatment, explaining the 1066 drugs prescribed clearly to the patient, and following the post-treatment process. The responsibilities and behaviours of physicians are to establish the correct diagnosis of the patient, to determine the most appropriate treatment options, to choose the treatment with or without drugs, and to determine the appropriate drugs for the treatment with drugs. At this point, it can be said that physicians are the most important component in the RDU process. Patients, pharmacists, other health personnel, the pharmaceutical industry, and official authority can be counted among the other components in the AIK process. Each component has an important role in the RUD process (Le Grand et al., 1999).

7. Pharmaceutical Waste

The drug must be used when and as needed. In addition to the economic dimension of people's drug use behaviours, there may be consequences such as incomplete treatment and drug poisoning, especially in young children. In 2007, approximately 14 billion dollars of drug sales were realized in our country and the annual drug expenditure per capita is approximately 200 dollars. This amount can be seen as low when compared to developed countries. However, considering that the ratio of drug consumption to national income is 1.75%, it will be seen that it is even higher than the US figure of 1.5%, and that drug expenditure is quite high compared to our income (TAF Preventive Medicine Bulletin, 2009: 8).

Since it constitutes a significant part of health expenditures, the rational use of drugs is of great importance in terms of more efficient use of limited resources. Rational drug use at the 1985 Nairobi meeting of the World Health Organization; It has been defined as "the ability to provide the patients with the appropriate time and dosage, at the lowest price and easily, according to their clinical findings and individual characteristics". Medicine in terms of public health; should be reliable, and affordable, can be provided to people from every income group, when needed, without delay, and it should be a product that the patient cannot choose on his own. In addition, it is a product that has a significant risk potential in the future due to the side effects it creates and that the consumer has the least knowledge about. The pharmaceutical industry is an industry branch that has developed rapidly in the last 40 years. However, drug-related wastes generated in areas where drugs are produced or consumed have not gained the importance they deserve in waste management, especially in developing countries. Hospitals, polyclinics, laboratories, veterinary clinics and other health units should carefully address this issue by establishing units for the management of drug-related wastes.

Medicines are produced in such a way that they are as durable as possible and their mobility in the liquid phase is high to be easy to drink and be stored for a long time. Due to these properties, the active substances and biotransformation products in the drug may accumulate in the ecosystem and cause various effects. Antibiotics, antibacterial drugs, painkillers and antipyretic drugs, beta-blockers, cholesterol drugs, cytostatic drugs, synthetic steroids, etc. are drugs that have been detected in the ecosystem through various research (Ruhoy IS, Daughton,2008).

It is a known fact that drugs accumulated in homes for various reasons cause accidental or voluntary poisoning. According to a study conducted in the United States, a pediatric patient is brought to the emergency services with the suspicion of poisoning every 7 minutes. 78,000 of these patients are children younger than 5 years old. The factors that cause poisoning in these children are; They are substances used for medicines and other purposes (cleaning, etc.) in homes. Many studies are questioning the existence and frequency of drug poisoning cases in our country (Aydınoglu H, Aygün AD, Güngör S, Turgut M,2000). For example; In a study, it was stated that 5077 pediatric poisoning cases reported from 38 health institutions in different regions of Turkey were most common in the 13 months - 4 age group, in males, and in the spring and summer months. Drugs are the first place as a cause of poisoning. In some regional studies, the first rank among the poisoning cases brought to the pediatric emergency outpatient clinic in the age group of 13 months - 4 years is drug-induced cases (Aji DY and İlter Ö, 1998). In a study conducted in Spain in 2007, 227 pharmacy customers returned 1176 packages of medicines that they did not use at home within 8 days. The total value of these drugs is 8,540 €, of which 6,464 € is paid by the state.

The Environmental Protection Agency (EPA: U.S. Environmental Protection Agency), HHS (Department of Health and Human Service) and the White House National Drug Control Regulation (ONDCP: The White House Office of The National Drug Control Policy) jointly prepared a regulation in 2007. have determined how citizens should dispose of these drugs in a way that prevents abuse of prescription drugs. The aim is to prevent the use of this group of drugs beyond their intended use and to prevent environmental problems. According to this regulation, drugs that are unused for some reason, out of need or whose shelf life has expired are respectively;

1- If there is a clear disposal instruction on the medicine package, it is followed. If there is no special knowledge, the medicine is never poured into the toilet

2. If there are no instructions, the drugs can be thrown into the household trash. However, before this process; Removed from their original boxes, the label and drug name on the medicine box is scratched or scribbled so that they cannot be read, it is mixed with tea, granulated coffee or cat litter and put in a sealed, sealable bag or can, the sealed bag/box is thrown into the trash, in case of doubt about the disposal of the drug, the pharmacist is consulted, etc.

In the United States, warning labels are placed on the packages of strong pain relievers and controlled drugs, and it is requested that these drugs be disposed of by pouring them into the toilets when necessary. However; It is thought that this practice, made for safety reasons, may cause pollution of surface and underground waters such as rivers and lakes.

8. Counterfeit Drug Production

People have used and continue to use drugs to protect their health or to regain their deteriorated health. This leads us to the conclusion that medicine and the need for medicine are almost as old as human history. It is stated that the most preferred

method in the treatment of diseases is drug therapy, with the developments in pharmaceutical technologies and many new drugs produced today. Therefore, it can be said that medicine is an important need for human beings. The fact that the cause of death of approximately one-third of the 57 million people who die every year in the world is the inability to access adequate medicine in the face of curable diseases is an indicator of how important this need is. However, while people use drugs to protect their health or to regain their deteriorated health, they also know that the drugs they use have some side effects. Since there are no drugs today without side effects or at least possible side effects, there is no drug that does not harm people or their health. Therefore, the drugs that people use to regain their deteriorated health can sometimes lead to further deterioration of their health and even death from time to time (Eren, 2009).

However, people decide whether to use the drug or not by comparing the benefit they hope to obtain from this drug when using the drug in question and the harm that will occur or may occur in their health in case of side effects. For people to make a comparison between the side effects of the drug on their health and the benefit they hope to obtain from the drug they use, they need to have a full knowledge of the side effects of that drug. Learning about the side effects of the drug is possible with the ongoing clinical drug research before and even after the drug is put on the market. However, sometimes even these drug studies are not sufficient to learn about the side effects of the drug; After the drug is put on the market and used by a large group of patients, it can be seen that the drug causes much more severe diseases than the disease it is hoped to cure.

In line with all these explanations, the production and sale of drugs are of great importance and include a process where many people may be responsible, from the manufacturer to the physician who prescribes the drug in question, and even to the pharmacist who gives this drug to the patient. In Turkish law, the act of making or selling drugs in a way that endangers the life and health of individuals is regulated as a crime in the Turkish Penal Code by the legislator and sanctioned. The crime in question is regulated in article 187 of the third part of the special provisions book of the Turkish Penal Code, titled crimes against society, the third part is titled crimes against public health. According to paragraph 1 of the said article, "Anyone who produces or sells drugs in a way that endangers the life and health of people is sentenced to imprisonment from one year to five years and a judicial fine". This situation constitutes a simple form of crime. In the second paragraph of Article 187, the qualified form of the crime is included. According to this; "If this crime is committed by a doctor or pharmacist or within the scope of a profession and art carried out based on official permission, the penalty to be imposed is increased by one-third".

The drug may be original, generic, counterfeit or counterfeit in terms of legal status. As a result of long research and clinical studies, drugs that have been proven to have a positive effect on a certain disease, based on a patented molecule and previously unique, are original drugs. Original drugs are protected within the patent period, within the scope of the patent right, and within the framework of legal provisions regulating patents and trademarks. Patent periods are stipulated in article 72 of the Decree Law on the Protection of Patent Rights No. 551. Generic drugs are drugs that are produced without the permission of the company that produced the original drug, contain the same active substance in the same amount as the original drugs, and have the same pharmaceutical form and formulation as the original drug after these patent periods have expired. Like original drugs, generic drugs must be licensed by the Ministry of Health to be put on the market and put up for sale. Since 2000, the Ministry of Health has sought to license generic drugs to have proven bioequivalence30 with the original drug. However, counterfeit drugs are drugs that are put on the market before the patent protection period of the original drug expires and have the same effect as the original drug. As can be seen, the counterfeit drug, like the generic drug, has bioequivalence with the original drug. However, just as the counterfeit drug is released to the market before the patent expiration of the original drug, its bioequivalence with the original drug is not subject to the control of the Ministry of Health. Therefore, while the generic drug is licensed and in compliance with the law; counterfeit medicine is illegal. On the other hand, products that contain different substances or different amounts than those written on the label, that does not contain active ingredients, or that have a fake label attached, are counterfeit medicines. Sawdust, corn flour, coffee and water-like substances are used in counterfeit medicines, and these drugs pose a danger to human life and health, as they do not treat existing diseases in patients or stop the development of the disease or protect healthy individuals from diseases.

Unsurprisingly, a product such as pharmaceuticals can be subject to mass counterfeiting. The price of real drugs is high, further increasing the profit margin on counterfeits and the global market of potential customers is huge. Furthermore, counterfeits can be difficult to detect. A patient is likely to attribute ineffective drugs to the severity of a disease, not the quality of the drug. Policies designed to promote domestic generic drug manufacturers may also allow for lower quality controls on exported drugs, allowing counterfeiters to sneak into the market. The European Commission reported that customs officials seized 2.7 million counterfeit drugs at EU borders; a year ago. The counterfeit pharmaceutical industry works in much the same way as counterfeiting designer bags or DVDs of Hollywood's latest blockbuster movies. It provides a superficially plausible imitation of the real product, produces large quantities of fakes, and relies on economies of scale to make a profit. Products are

often distributed by the same criminal agents who deal with other counterfeit goods. The difference is that counterfeit drugs can carry a human price tag. For much of the past decade, lifestyle drugs - erectile dysfunction drugs, pain relievers and anti-anxiety drugs like Valium - were the most common knockouts, especially in wealthy countries. But in the last few years, counterfeiters have turned to much more life-threatening counterfeit pharmacology, producing drugs used to treat cancer, HIV/AIDS, and serious heart conditions. As many as 1 million people die annually from buying these counterfeit products, mostly in developing countries, but their numbers are increasing in rich countries as well. Last year, at least 95 Americans died from allergic reactions to Chinese-made heparin, a drug used to prevent blood clots. Sources at the U.S. Food and Drug Administration tell me that contaminated heparin is almost certainly counterfeit. Some counterfeit drugs are good copies of branded drugs, hygienically made with the right ingredients in the right proportions. They sure do violate intellectual property rights, but they are not inherently dangerous as long as they are perfect copies. Unfortunately, for most counterfeiters, the motivation is profit, not reliable products.

9. DNA Barcoding Technology

A phrase that has been often used in the literature is "DNA barcoding" (Figure 1) This procedure relies on the usage of a typical mitochondrial DNA region that is 600–700 base pairs long to quickly, accurately, and automatically identify species. (PDN, 2005: 54). When DNA barcoding is analyzed, it becomes clear that it is not a novel idea. In a 1993 study that received little attention from the scientific world, the phrase "DNA barcode" was first used (De et al., 1995: 61). In actuality, the expression of accomplishing species identification using molecular techniques was a more ancient idea than the Sanger sequencing method. However, the genuine "golden age" of DNA barcoding began in 2003, and since then, there have been a lot more publications on the topic, with more than 1000 articles published as of this writing. DNA barcoding has potential uses outside taxonomists. This technique has established itself in the literature as a versatile instrument that may be applied to a variety of disciplines, including forensic sciences, biotechnology, the food business, animal nutrition, genetic diversity, and species discrimination. A short portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene, or roughly 655 base pairs, has been effectively used in studies so far to obtain 98–100% identification levels in several species belonging to complex groups like birds, fish, and butterflies. Many mysterious species that were previously thought to be one species could be found utilizing the barcoding technique. Barcoding allows for quick and accurate species identification, and it also makes it simpler to evaluate evolutionary relationships between various groupings.

The DNA barcoding technique mainly rests on a single premise and shows species-specific DNA profiles. According to this assumption, tiny regions of an organism's genome with different DNA sequences can serve as biological barcodes to identify any living entity at the species level. Therefore, it is conceivable to develop a universal species identification key that will allow these species to be recognized by comparing the DNA sequences of unidentified species with the DNA sequences in DNA barcode databases. The presumption that there is less diversity within a sequence than there is between species forms the basis for the usage of short DNA sequences. The process involves isolating the DNA from tissue samples acquired from living things, amplifying the desired region with this DNA using PCR, and analyzing the amplified region's DNA sequence. These methods produce DNA sequences that are stored in data banks as barcodes for identifying species (Aravind et al., 2007: 92). The main feature that distinguishes DNA barcoding from other methods is that it has the principle of a universal biological identification system for all animal species using a single gene region. The 655 base pair region from the 5' end of the COI gene has been determined as the most suitable region for DNA barcoding since it can be amplified with primers that can be designed for many organisms from different branches. A gene region needs to possess three characteristics to be as helpful as a DNA barcode. They are as follows:

1. with high genetic diversity and the ability to distinguish between species,
2. Possessing protected end regions that can be amplified by universal primers appropriate for species from a broad taxonomic range,
3. Possessing a brief sequence length that will not interfere with DNA extraction and PCR. The COI gene has been acknowledged as a standardized barcoding area with species-level discrimination capability in animals as a result of all these characteristics.

Mixing DNA with Ink

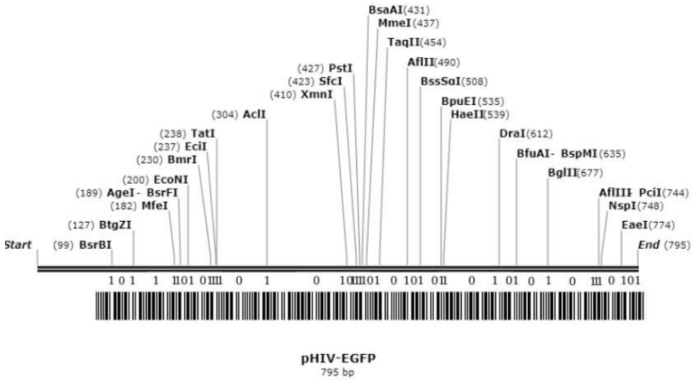

Figure 1. Restriction sides

A DNA-containing ink is offered that barely breaks down when exposed to environmental stimuli like heat, acid, or alkali.

Additionally, a technique for quickly evaluating DNA in an ink composition is offered. Because single-stranded DNA is less physically stable and more expensive than double-stranded DNA, double-stranded PCR-amplified DNA will be utilized to incorporate it into the DNA ink. A colourless transparent pigment and three mg of double-stranded DNA will be combined to create an invisible ink, and an IR colour former will be added to make it simple to see the printed mark. Dummy DNA will also be included to make it more challenging for anyone not familiar with the primer sequences to examine the DNA-ID sequence.

10. DNA Ink Stability and Efficiency Test

Heat test: After the ink is kept at a dry temperature of 150 degrees for 10 minutes, the detection of DNA degradation will be carried out with 1% agarose gel electrophoresis at 120 volts for 1 hour, then subjected to these temperatures separately for 0 minutes, 1 minute, 5 minutes and 10 minutes, to the breaking rate will be looked into.

Acid test: It will be kept separately for 0 minutes, 10 minutes, 30 minutes, 1 hour, and 2 hours at 20.25 degrees in 2% sulfuric acid and will be run separately with 1.5% agarose gel electrophoresis at 120 volts for 10 minutes, and then decomposition by comparison with DNAINK ink that has not been exposed to any acid rate will be calculated.

Alkali test: After being carried out at 120 volts for 10 minutes with 1.5% agarose gel electrophoresis to be kept separately for 0 minutes, 10 minutes, 30 minutes, 1 hour, and 2 hours at 20.25 degrees in an environment with 1% sodium hydroxide, the disintegration rate is compared with the ink that has not been exposed to alkaline will be calculated.

Paraffin test: It will be run at 120 volts for 10 minutes with 1.5% agarose gel electrophoresis, which will be kept separately for 0 minutes, 10 minutes, 30 minutes, 1 hour, and 2 hours in melted paraffin at 100.110 degrees, and then compared with ink that has not been exposed to paraffin.

Soap test: 10% soap will be proportioned with DNAINK that has not been exposed to the soap after 10 minutes at 120 volts with 1.5% agarose gel electrophoresis, which will be kept separately at 20.25 degrees for 0 minutes, 10 minutes, 30 minutes, 1 hour, and 2 hours.

Alcohol test: It will be compared with ink that has not been exposed to an alcoholic environment after being carried out for 10 minutes at 120 volts with 1.5% agarose gel electrophoresis, which will be kept separately at 20.25 degrees for 0 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours in 100% ethanol environment.

Sunlight test: It will be kept in a sun-exposed window for 1 hour, 2 hours, 1 day, 1 week and 1 month, and will be run with 1.5% agarose gel electrophoresis at 120 volts for 10 minutes and then compared with ink that has not been exposed to sunlight.

By performing these tests, the resistance of the DNA ink produced against active substances, the breakage and deterioration time and efficiency will be measured and its stability will be known.

11. DNA Barcode Sequencing and Analysis

After making the DNA ink and subjecting it to active substance tests, Sanger sequencing is aimed. The target after this sequencing is to test whether the barcode system is working by reanalyzing the ratios of the distances of the regions that the restriction enzymes can cut in the DNA ink. The pHIV-EGFP plasmid DNA will be used here for the effectiveness and testing of the ink and barcode technology. The pHIV EGFP plasmid DNA to be used is confirmed in the diagram below.

12. Discussion

Our ultimate goal will be achieved thanks to DNA Barcoding Technology, which has already begun to be developed. It will be understood how long the protection will be provided by continuing various tests to maintain the stability of the DNA Ink. For this, in the transformation experiments, which is the first step, colonies were observed on dry and liquid plates, one step closer to the desired result, and more colonies are expected with different experiments. After completing this process, it is aimed to make an important contribution to the field of health thanks to ink stability and barcoding technology. To talk about the objectives, within the scope of this project, with the DNA-based ink used in the packaging of drugs, thanks to the DNAINK method being developed, it is possible to easily detect the deterioration of drugs, to track and monitor the deterioration of drugs in case they lose the appropriate environmental conditions, to determine whether the cold chain transfer is interrupted, to ensure that the drug is used in suitable environmental conditions. It is possible to track whether it is stored or not and to ensure its authenticity, with the barcoding technology being developed, whether the drug is produced in the right place or not. Among the final targets are to contribute to the creation of added value by reducing the waste in medicines, to prevent the harm that spoiled medicine can cause to human health and to contribute to the conscious use of medicines. By means of barcoding technology, to prevent and prevent factories that produce fake drugs in the wrong production place from affecting human health to a large extent (heat and light targets), experimentally measurable things, experimental measurements of factors such as heat, light and humidity that affect the

deterioration of drugs, to what extent they affect the deterioration of the drug. to determine what it does and to implement it within the scope of this project.

References

Acar A, Yeğenoğlu S. Türkiye'de İlaçta Patent. Ankara Eczacılık Fakültesi Dergisi, 2004; 33(4) 269-85.

Açıkel CH. Akılcı İlaç Kullanımı. TAF Prev Med Bull. 2003; 1(11): 5-6.

Aji DY, İlter Ö. Türkiye'de Çocuk Zehirlenmeleri. Türk Pediatri Arşivi 1998; 33:154-158.

Akıcı, A. ve KALAÇA, S. (2013). "Topluma yönelik akılcı ilaç kullanımı". Sosyal Güvenlik Kurumu Başkanlığı, SGK Yayın No: 93, Ankara

Ay, E. (2018), İlaç Sektöründe Lojistik Yönetimi ve Tersine Lojistik. Dönem Projesi, Sosyal Bilimler Enstitüsü, Pamukkale Üniversitesi.

Arnot DE, Roper C, Bayoumi RA. 1993. Digital codes from hypervariable tandemly repeated DNA sequences in the Plasmodium falciparum circumsporozoite gene can genetically barcode isolates. Molecular and Biochemical Parasitology. 61:15-24.

Aravind K, Ravikanth G, Shaanker RU, Chandrashekara K, Kumar ARV, Ganeshaiah KN. 2007. DNA barcoding: An exercise in futility or utility? Current Science. 92(9):1213-1216.

Boğ, M. (2005). İlaç sektöründe lojistik uygulamalar (Doctoral dissertation, Fen Bilimleri Enstitüsü).

Busfield J. Assessing the overuse of medicines. Soc Sci Med. 2015;131:199–206.

Butler, N.J. The microbiological deterioration of cosmetics and pharmaceutical products. In Biodeterioration of Materials 269 (1968) (Elsevier Publishing Co., Amsterdam, London and New York).

Centers for Disease Control and Prevention, (2003), "Guidelines for Maintaining and Managing The Vaccine Cold Chain", Morbidity and Mortality Weekly Report, https://www.ncbi.nlm.nih.gov/pubmed/14574279 (Erişim Tarihi: 11.05.2020).

Chen X, Smith LM, Bradbury EM (March 2000). "Site-specific mass tagging with stable isotopes in proteins for accurate and efficient protein identification". Analytical Chemistry

Chongqing Key Laboratory of Multi-scale Manufacturing Technology, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing 400714, China

European Environment Agency. Pharmaceuticals in the environment: results of an EEA Workshop. Copenhagen: EEA; 2010 (EEA Technical Report No 1/2010).

"Fluorescent labeling of biomolecules with organic probes - Presentations - PharmaXChange.info". 29 January 2011

Georgina,The structure of DNA,2019

Hayrettin Eren, "İlaçların Onaylanması ve Piyasaya Sürülmesinden Dolayı Devletin Sorumluluğu", İlaç Hukuku, Editörler: Murat Şen/Ahmet Başözen, Erciyes Üniversitesi Hukuk Fakültesi I. Sağlık Hukuku Sempozyumu, 08-09 Mayıs 2009, Kayseri, 283.

Hertz, S. ve Alfredsson, M. (2003). Strategic development of third party logistics providers. Industrial Marketing Management, 32(2), 139-149.

Holloway KA. Combating inappropriate use of medicines. Expert Rev Clin Pharmacol. 2011;4(3):335–48.

Hebert PDN, Gregory TR. 2005. The Promise of DNA Barcoding for Taxonomy. Systematic Biology. 54(5):852-859.

Hatchett,The medicines refrigerator and the importance of the cold chain in the safe storage of medicines,London,2017

İpekçi E. Tanyaş M. Soğuk Zincir Lojistiği Uygulamaları ve Türkiye'de Soğuk Zincir Lojistiğinin Swot Analizi, 2021.

Julian, Silvan, Kunal, Wendelin, Yaniv, A DNA-of-things storage architecture to create materials with embedded memory, Nature Biotechnology, 2020.

Johnson FH, Shimomura O, Saiga Y, Gershman LC, Reynolds GT, Waters JR. 1962. J. Cell. Comp. Physiol. 60:85–103

Kartoglu, Ümit ve Milstien, Julie (2014), Tools and Approaches to Ensure Quality of Vaccines throughout the Cold Chain, Expert Review of Vaccines, 13(7), s. 843-854.

Karakoç, H.D. (2005). İlaç Sektöründe Fiyat Rekabeti, Uzmanlık Tezi, Rekabet Kurumu, Ankara.

Le Grand, A., Hogerzeil, H. V., ve Haaijer-Ruskamp, F. M. (1999). "Intervention research in rational use of drugs: a review". Health policy and planning, 14(2), 89-102.

Masaki, Development of Biometric DNA Ink for Authentication Security,PubMed, November 2004

Morin JG, Hastings JW. 1971. J. Cell. Physiol. 77:313–18

National Audit Office. Prescribing costs in primary care. London: National Audit Office; 2007 (www.nao.org.uk/ publications/0607/prescribing_costs_in_primary_c.aspx, accessed 19 September 2016).

Physiol. 77:313–18 4. Morise H, Shimomura O, Johnson FH, Winant J. 1974. Biochemistry 13:2656– 62

Rajesh ,Richa,Ashok,Madhu, Molecular Mechanisms of Ultraviolet Radiation-Induced DNA Damage and Repair, 2010 Dec

Rajeshwar,Donat P, UV-induced DNA damage and repair: a review, 2002 Apr. Physiol. 77:313–18 4. Morise H, Shimomura O, Johnson FH, Winant J. 1974. Biochemistry 13:2656– 62

Shan,Zev, Rapid Short-Read Sequencing and Aneuploidy Detection Using MinION Nanopore Technology, Genetics, Volume 202, Issue 1, 1 January 2016

Shimomura O, Johnson FH, Saiga Y. 1962. J. Cell. Comp. Physiol. 59:223– 39

Schneller, Smeltzer. Strategic Management of the Health Care Supply Chain (2006)

Thomas F, Depledge M. Medicine 'misuse': implications for health and environmental sustainability. Soc Sci Med. 2015;143:81–7

Timur, M. N. Tedarik zinciri-temel kavramlar. M. N. Timur & G. S. Çekerol (Edt.), Tedarik zinciri yönetimi içinde (s. 2-22). Eskişehir: Anadolu Üniversitesi Açıköğretim Fakültesi Yayınları. (2013)

Tengilimoğulu D. ve Yiğit V. (2013). Sağlık İşletmelerinde Tedarik Zinciri ve Malzeme Yönetimi. Nobel Akademik Yayıncılık, İstanbul.

Yanxiao,Yuechuan,Cuifeng,Deqiang, Nanopore-based Fourth-generation DNA Sequencing Technology, February 2015

Walker, H. W. and Ayres, J. C. Yeasts as spoilage organisms. In The Yeasts 3 463 (1969) (Academic Press, London and New York

Wen, Zhi - Liao Huchang - Ren Ruxue - Bai Chunguang - Zavadskas Edmundas Kazimieras - Antucheviciene Jurgita - Al-Barakati Abdullah, (2019), "Cold Chain Logistics Management of Medicine with an Integrated Multi-Criteria Decision-Making Method", International Journal of Environmental Research and Public Health, 16(23), 4843.

Nei M, Kumar S (2000). Molecular Evolution and Phylogenetics. 1st ed. New York, NY, USA: Oxford University Press.

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Review Article

©Gene Editing
(2022) 03: Pages 30-34.
DOI: 10.29228/genediting.66929

Expression of Axolotl P53 Gene May Increase Cancer Resistance

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Received: 09.12.2022 **Accepted/Published Online**: 21.12.2022 **Final Version**: 25.12.2022

Abstract: This review is about developing a cancer treatment using a gene therapy approach focused on the p53 gene, which is responsible for one in six deaths worldwide. When we look at the origins of cancer, we observe tumour suppressor genes that control regular cell activity being expressed and then mis-regulated by genetic alterations. Reversing cancer cell tumorigenicity gives vital hints for cancer therapies. Understanding the molecular pathways involved in the loss of tumour suppressor gene activity is a crucial obstacle for cancer treatments. During the project process, axolotl (Ambystoma mexicanum) variations of the p53 gene, known as the cancer resistance gene, were focused on. This is because mutations in the human p53 gene are the most common cause of cancer and increase cancer resistance in the future. The axolotl is an important amphibian with the ability to regenerate. In addition to its regeneration feature, it shows high resistance to cancer formation. Axolotl adults do not undergo metamorphosis and live in water. Despite this situation, it has been observed that it is resistant when exposed to carcinogens. The human p53 gene contains mutation sites in the axolotl p53 gene, which differs from the human p53 gene by 38 amino acids. If the data collected at the end of the study yields the desired findings, it is hoped to develop products with more in-depth research and conduct genetic treatment studies in cancer-cancer resistance.

Key words: Axolotl, P53 gene, Cancer resistance, Recombinant DNA

1. Introduction

Axolotls, the real name Ambystoma mexicanum, belong to the urodele amphibians' group (Villiard, 2007). Amphibians are a group that undergoes a metamorphosis. The larvae emerge from the eggs laid in water and develop into adult forms with the necessary features to survive on land. When looking at axolotls, axolotls do not turn into adult forms with the mentioned features. In other words, they do not undergo metamorphosis. For this reason, axolotls are neotenic creatures (Springhetti, 2022). This feature has aroused serious interest in axolotls in the scientific world and it has started to be used as a model organism in laboratories (Suleiman, 2019). In line with the research on it, the regeneration feature of axolotls was also emphasized. The reason for this is that the regeneration feature seen in amphibians including axolotls is much more developed in axolotls. While other amphibians have limited regeneration capabilities, axolotls can regenerate all parts of their body, such as their entire limbs and tail, upper and lower jaw, brain, spinal cord, and myocardial muscle (Suleiman, 2019). The ability to regenerate complex structures in this way makes axolotls an important model for cancer research as well as being used in studies to study and understand regeneration. Axolotls are also cancer-resistant creatures. They showed resistance to tumorigenesis when exposed to carcinogens, and regression of tumour cells was observed in another experiment (Ingram, 1971). In line with these data, a connection was established between the enormous regeneration feature of axolotls and their resistance to cancer.

Axolotl as Regeneration Ability

The functional restoration process after damage in the living thing is called "regeneration". The complete realization of regeneration depends on the proliferation of stem cells and differentiable cell types. For the process to take place in a controlled manner, the activation of death pathways induced by injury is necessary. With the activation of death pathways, damaged cells are destroyed. Afterwards, the blastema tissue, which plays a role as the main character in the regeneration process, is formed. The blastema performs differentiation with the coordination of the different cells it has. As a result of differentiation, a small-sized version of the damaged structure (the structure to be regenerated) is created. This created structure continues to grow until it reaches the size of the original damaged structure.

Signalling pathways of JNK, TLR, JAK/STAT, Hippo/YAP, Wnt/ß-catenin for the complete regeneration process; Expressions of epidermal growth factor (EGF), transforming growth factor ß (TGFß), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) need to be regulated.

These factors play a role in dedifferentiation, redifferentiation, cell division, and growth processes (Demircan, 2017). Regeneration is observed in many plants and animals. However, this feature does not occur at the same level in all living things. Regeneration levels differ between living things. Invertebrates such as sponges, and cetaceans have a high level of regeneration. However, this level decreases from fish to mammals in vertebrates (Alvarado, 2004). In mammals, regeneration usually occurs at the tissue level. The regeneration capacity of salamanders is quite high. Salamanders can regenerate amputated or damaged limbs and organs. The most important of these creatures are axolotls. Since axolotls do not undergo metamorphosis and therefore adult individuals show similar features to the embryonic developmental period, they have higher regeneration characteristics than other salamanders. These creatures can regenerate their limbs, heart, brain, spinal cord, and other internal organs (Demircan, 2017).

The jaw and other appendages regenerate with the formation of mesenchymal growth and blastema in the amputation plane, which is one of the regeneration mechanisms (Brockes, 1997). Mesenchymal cells in the blastema divide to differentiate to reconstruct the lost structure with morphogenesis and amputation. Urodele amphibians, on the other hand, can locally affect the regeneration of differentiated tissues as a response to tissue or limb loss. An example of this is differentiated cells of the iris epithelium or limb mesenchyme (Brockes, 1997). These differentiated post-mitotic cells can re-enter the cell cycle and lose their differentiated character in both lens and limb regeneration (Brockes, 1997). This event is reversible, with cells stopping ocular progenitor cells or limb blastema cells after several divisions and differentiation into the lens or limb mesenchyme (Brockes, 1997).

The axolotl limb regeneration model is amongst the most studied regeneration models and the fact that many genes and signalling pathways are conserved between humans and axolotls makes this organism an ideal model in therapeutic research. Even though the axolotl genome is 10 times larger than the human genome at 32 gigabases (GB), it roughly encodes a similar number of proteins as humans with extensive conservation of synteny (blocks of order within two sets of chromosomes that are being compared with each other) between Ambystoma, chicken, and human, and a positive correlation between the length of conserved segments and genome size. Several other reasons make the axolotls very attractive models for research, including their high reproductive rate and low maintenance requirements in the laboratory. In comparison to the newt (Notophthalmus viridescence), axolotls are much easier to breed. The fact that axolotls are sexually mature and yet maintain a larval state could prove to be pivotal to cancer research.

Relationship Between Regeneration, Cancer Formation, and Axolotl

Humans have limited tissue cell differentiation. The human body cannot regenerate complex structures. Fingertips containing the liver and distal/terminal phalanges are not included. The human body can regenerate these structures. This is included in the "Limited regenerative feature". An example of this property is the ability to repair tissues such as bone, peripheral nerves, and skin cells under UV exposure. Compared to the regenerative properties of some animals, this feature is not as efficient and faultless as they are. With such a difference between living things with highly developed regeneration features and humans, signalling pathways effective in regeneration, embryonic development and cancer formation are seriously protected on both sides. Among the important points in cancer formation, the EMT (epithelial-mesenchymal transition) mechanism, which is vital for embryonic development, is also very important for regeneration and wound healing (Suleiman, 2019). This mechanism is the transformation of epithelial cells and mesenchymal cells into each other. In this transformation process, cells lose some of their properties and take on the characteristics of the cell they are transformed into. For example, while epithelial cells transform into mesenchymal cells, they lose their polarity and cell-cell adhesion properties but gain the characteristics of migration, proliferation, and differentiation. In line with these features, the EMT mechanism is very important in cancer metastasis. For this reason, any irregularity or mistake that occurs in this mechanism can trigger the formation of cancer.

There are both differences and similarities between proliferation episodes and neoplasia in animal tissues. On the other hand, the regeneration feature in urodele amphibians is associated with a high level of resistance to tumour formation (Brockes, 1997). Observation of tumour formation in an animal with low regeneration ability suggested that this was the result of blastema formation (Brockes, 1997). This situation, which was in the pre-oncogene age, together with the "regeneration" feature created a different perspective on cancer. With this perspective, in addition to the classical idea of tumorigenesis (mutations in dividing progenitor cells), tumors also connect the balance between proliferation and differentiation and regeneration issues (Brockes, 1997). When we look at the characteristics of axolotls, their prominent features are their long lifespan of 25 years in captivity, their wide regeneration ability, and their resistance to tumour formation.

The onset of the regeneration process in axolotls begins with limb injury. Immediately after injury, membrane keratinocytes migrate to the injured area in layers. Thus, it covers the wound area. Keratinocytes reaching the injured area proliferate thus forming the wound epidermis and the wound is innervated

within a few days. Afterwards, the apical educational cap (AEC) is created. With the AEC, the adaptation process of the regenerating limb becomes easier. Pro-regenerative signalling molecules are provided by AEC (Boilly, 2017). These pro-regenerative signalling molecules consist of FGF, TGFß, IGF, BMP, and WNT, which are necessary for limb growth in the regeneration process in salamanders (Boilly, 2017). The blastema is formed because of the interaction between the AEC and the underlying tissue. AEC contributes to blastema formation by inducing cellular differentiation. This differentiation occurs with the re-entry of post-mitotic differentiated cells into the cell cycle.

Many genes have been associated with differentiation in proteomic studies. Adult somatic cells express the transcription factor genes KLF4, Sox2, and c-myc. Thus, pluripotency is reprogrammed (Boilly, 2017). Cirbp and kazald1 proteins played a role in blastema formation. In the process of blastema formation, conditions such as denervation or deviation of nerves and macrophage depletion disrupt the interaction of the epidermis and the underlying cells, so that regeneration does not occur (Boilly, 2017). This situation has been similarly described between cancer cells and neurons. Considering this situation with the logic of feedback, the interaction induces neuronal growth by cancer cells, and then neuronal cells induce cancer metastasis. This mechanism has been observed in many types of cancer, such as basal cell carcinoma, and prostate and gastric carcinomas. In addition, it is known that nerves and some neurotransmitters such as BMP2 and FGF are directly involved in regeneration and cancer microenvironments (Suleiman, 2019).

p53 Mechanism on Cancer and Regulation

The idea that there is a relationship between the unlimited regeneration ability of urodele amphibians and their high level of cancer resistance has made them model animals used for cancer studies (Villiard, 2007). In this case, when the genes that take an active role in both cancer and regeneration processes are examined, it is seen that the p53 gene is an ideal research subject. The p53 tumour suppressor gene can affect the ageing rate and modulate regeneration in both mammals and drosophila.

The p53 gene is a gene that encodes the Tp53 protein. Mutations in the p53 gene are the cause of approximately 85% of common cancer types today. Because the p53 gene is a tumour suppressor gene, also known as the "guardian of the genome". The p53 gene is also a transcription factor, so the p53 gene can affect the transcription process. The p53 gene has an important role in the cell cycle. It provides intervention if there is any problem at the checkpoints in the cell cycle process. Any problem in the DNA is detected during the G1 phase of the cell cycle. This detected error induces the expression of the p53 gene and the p53 level increases. P53 induces the secretion of p21 protein, and p21 protein binds to the CDK complex and blocks this complex. Thus, the cell cannot switch to the next cycle and the cell cycle is stopped in the G1/S phase. After the cell cycle is stopped, p53 induces the genes necessary for the correction of the detected DNA damage and DNA repair begins. If the damage cannot be repaired, p53 decides the apoptosis of this cell and initiates the apoptosis process. The p53 gene is regulated by the Mdm2 gene. Since a mutation in the p53 gene will prevent this control mechanism in the cell cycle process, the damaged DNA will not undergo apoptosis and will continue with the cell cycle, thus cancer formation will occur with uncontrolled proliferation. As can be understood, the p53 gene has two basic mechanisms. One is the repair mechanism and the other is the apoptosis-cell death mechanism. If the damaged area can be repaired, the repair mechanism is activated, and repair is made. However, if the repair cannot be made, this time the apoptosis mechanism is activated, and the cell is killed.

Selective pressures associated with certain lifestyles can alter the properties and signalling pathway of p53. For example, Spalax living in hypoxic conditions does not activate p53 in response to hypoxia (Ashur-Fabian, 2004), while ground squirrels have lower p53 levels in their nuclei during hibernation than in summer animals (Fleck, 2005). The discovery of p53 in model organisms such as Drosophila and *C. elegans* has revealed that p53 has evolved concerning the regulation of apoptotic pathways in response to DNA damage (Derry, 2001). Such examples and studies demonstrate the ability of the p53 system to adapt to different lifestyles. However, the idea can be formed that different variations of p53 can be discovered in nature.

Axolotl p53 Gene and Human p53 Gene

The focus is on the p53 gene of axolotls, which have high levels of regeneration and cancer resistance. The axolotl p53 gene is like the human p53 gene. At the same time, the axolotl p53 gene has an open reading frame of 387 amino acids (Villiard, 2007). In addition, 38 amino acid changes in the Axolotl protein correspond to mutations present in human cancers or that affect protein stability or change sites important for post-translational modifications (figure 1). However, T155, V157 and R283 are usually mutated in human cancers. Changes in the T155A and V157L regions are common to human p53 mutant proteins and axolotl p53 (Thukral, 1995). Axolotl p53 can activate the transcription of human p53 target promoters in human cells. Another feature of the axolotl p53 gene is that p53 signalling is required in limb regeneration (Villiard, 2007).

The similarities between the axolotl and human p53 gene

The hypothesis of "transgenic axolotl p53 gene increases cancer resistance in human cell lines", Axolotl p53 gene and human p53 gene fasta format were obtained using the ensembl.org genome

database. Afterwards, the fasta formats of 12 different primate p53 genes were obtained from the ensembl.org genome database to obtain similarity maps between the p53 genes of primates with human genome similarity and the axolotl and human p53 genes. For comparison, multiple sequence alignment of p53 gene fasta formats was performed using the genome.jp tool CLUSTALW base. As a result of using this tool, a p53 gene phylogenetic tree of 12 different species was obtained **(Figure 1)**.

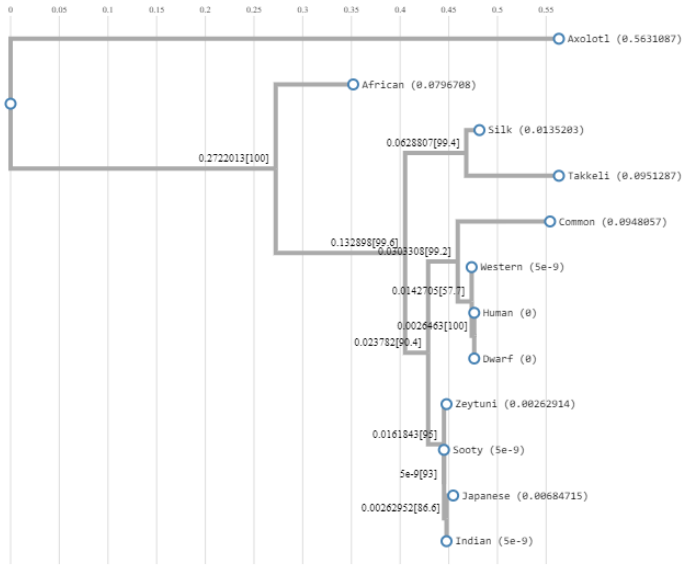

Figure 1: Phylogenetic tree of p53 genes from 12 different species

2. Discussion

When looking at the origin of cancer, it is seen that there is the expression of tumor suppressor genes that control normal cell function and the mis regulation of these genes by genetic changes. Understanding the molecular mechanisms involved in the loss of tumor suppressor gene function represents a significant hurdle for cancer treatments and reversing cancer cell tumorigenicity provides essential clues for cancer treatments. During the research process, axolotl (Ambystoma mexicanum) variations of the p53 gene, known as the cancer resistance gene, were focused on. This is because mutations in the human p53 gene cause 80% of common cancer types, and in line with the data obtained in the future, methods aiming to increase cancer resistance through the p53 gene have been developed. At the same time, selective pressures associated with certain lifestyles can alter the properties and signalling pathways of p53. Appropriate modulation of the p53 system to provide a long lifespan, tissue regenerative capacity, and efficient tumor suppression is another reason for choosing this gene. These studies reveal the surprising ability of the p53 system to adapt to different lifestyles and suggest that more interesting variations may still be found in nature.

The axolotl is an important amphibian with the ability to regenerate. In addition to its regeneration feature, it shows high resistance to cancer formation. The existence of many conserved genes and signalling pathways between humans and axolotls has brought this organism into focus in therapeutic research. Many studies have linked the cancer resistance and regeneration ability observed in axolotls. The axolotl p53 gene (GENBANK accession number DQ848588) has an open reading frame of 387 amino acids with significant sequence similarity to the human p53 gene. In addition, 38 amino acid changes in the Axolotl protein correspond to human cancers or affect protein stability or change sites important for post-translational modifications.

Along with the development of high technology, the developments in the field of Molecular Biology have also been enriched. Specifically, in cancer studies, the focus has been on identifying the mechanisms of genes, transcription factors, and signalling pathways that regulate regeneration and cellular events that regulate cancer.

Thanks to technologies such as CRISPR, RNA-seq, and recombinant DNA transfers in the field of Molecular Biology, it has been and will be very supportive in the identification and interpretation of these mechanisms and proteins. Although much is known about the regeneration mechanisms in axolotls, research on the links between cancer and regeneration will gain momentum with the development of molecular biology techniques. By understanding the processes governing cellular proliferation, differentiation, cell cycle arrest, and redifferentiation using animal models such as axolotls through genetic engineering in cancer treatment, more light can be shed on the links between regeneration and cancer to explore new therapeutic approaches.

References

Amer, M. H. (2014). Gene therapy for cancer: present status and future perspective. Molecular and Cellular Therapies, 2(1), 27.

Ashur-Fabian, O., Avivi, A., Trakhtenbrot, L., Adamsky, K., Cohen, M., Kajakaro, G., Joel, A., Amariglio, N., Nevo, E., Rechavi, G. (2004). Evolution of p53 in hypoxia-stressed Spalax mimics human tumor mutation. Proc Natl Acad Sci U S A, 101(33):12236-12241.

Brockes, J.P. (1998) Regeneration and cancer. Biochim Biophys Acta 1377(1):M1-11.

Derry, W.B., Putzke, A.P., Rothman, J.H. (2001) Caenorhabditis elegans p53: role in apoptosis, meiosis, and stress resistance. Science 294(5542):591-595.

Fleck, C.C., Carey, H.V. (2005) Modulation of apoptotic pathways in intestinal mucosa during hibernation. Am J Physiol Regul Integr Comp Physiol, 289(2):R586-R595

Ingram AJ. (1971). The reactions to carcinogens in the aksolotl (Ambystoma mexicanum) in relation to the 'regeneration field control' hypothesis, 26(3) 425-41.

Murphy, P.J., Galigniana, M.D., Morishima, Y., Harrell, J.M., Kwok, R.P., Ljungman, M., Pratt, W.B. (2004) Pifithrin-alpha

inhibits p53 signaling after interaction of the tumor suppressor protein with hsp90 and its nuclear translocation. J Biol Chem 279(29):30195-30201.

Suleiman, S., Wismayer, P. S., Agius, J. C., 2019. The aksolotl model for cancer research: a mini-review. JBUON, 24(6), 2227-2231.

Thukral, S.K., Lu, Y., Blain, G.C., Harvey, T.S., Jacobsen, V.L., (1995) Discrimination of DNA binding sites by mutant p53 proteins. Mol Cell Biol 15(9):5196-5202

Villiard, É., Brinkmann, H., Moiseeva, O., Mallette, F. A., Ferbeyre, G., & Roy, S. (2007). Urodele p53 tolerates amino acid changes found in p53 variants linked to human cancer. BMC Evolutionary Biology, 7(1), 180.

Vogelstein, B., Lane, D., Levine, A.J. (2000) Surfing the p53 network. Nature, 408(6810):307-310.

Vieira, WA, Wells, KM ve McCusker, CD (2020). Yenilenme ve Yaşlanma için Axolotl Modelindeki Gelişmeler. Gerontoloji , 66 (3), 212–222. https://doi.org/10.1159/000504294

Demircan, T., Avşaroğlu, M. E., Öztürk, G. ve Keskin, İ. (2017). Aksolotl rejenerasyonunun farklı evrelerinde miRNA profilinin bulunması ve bulunan miRNA'ların görevlerinin in-siliko analizi. Haydarpaşa Numune Eğitim ve Araştırma Hastanesi Tıp Dergisi, 57(3), 125-134. https://dx.doi.org/10.14744/hnhj.2017.50251

Springhetti, S., Bucan, V., Liebsch, C., Lazaridis, A., Vogt, PM, & Strauß, S. (2022). Axolotl (Ambystoma mexicanum, Amex) Telomeraz Ters Transkriptazın (Amex TERT) Tanımlanması ve Karakterizasyonu. Genler , 13 (2), 373. https://doi.org/10.3390/genes13020373

Ingram AJ. The reactions to carcinogens in the axolotl (Ambystoma mexicanum) in relation to the "regeneration field control" hypothesis. J Embryol Exp Morphol. 1971 Dec;26(3):425-41. PMID: 4111936.

Sánchez Alvarado A. (2004). Regeneration and the need for simpler model organisms. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 359(1445), 759–763. https://doi.org/10.1098/rstb.2004.1465

Brockes J. P. (1998). Regeneration and cancer. Biochimica et biophysica acta, 1377(1), M1–M11. https://doi.org/10.1016/s0304-419x(97)00029-2

Boilly, B., Faulkner, S., Jobling, P., & Hondermarck, H. (2017). Nerve Dependence: From Regeneration to Cancer. Cancer cell, 31(3), 342–354. https://doi.org/10.1016/j.ccell.2017.02.005

