REVIEW
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Kistik Fibroziste Yeni Terapötik Yaklaşımlar

New Therapeutic Approaches in Cystic Fibrosis

Dolunay Merve FAKIOĞLU[1], Beril Altun[2]
[1]Gazi University Faculty of Pharmacy, Department of Clinical Pharmacy
[2]Gazi University Faculty of Pharmacy, Department of Pharmaceutical Toxicology

Corresponding Author
Beril Altun
berilaltun@gmail.com
+905068201282
orcid.org/0000-0003-3083-9854
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ÖZET
Kistik Fibrozis (KF), Kistik Fibrozis Transmebran İletkenlik Düzenleyicisini (KFTR) kodlayan KFTR genindeki farklı mutasyonların neden olduğu kalıtsal, multisistemik bir hastalıktır. Kistik fibrozis, esas olarak hava yollarındaki anyon transportunun ve mukosiliyer klerensin bozulması sonucu gelişen pulmoner disfonksiyon ile karakterizedir. Mortalite, genellikle bronşektazi, bronşiyollerin tıkanması ve erken dönemde ilerleyici solunum fonksiyon bozukluğundan kaynaklanır. Son on yılda, küçük molekül yaklaşımı, iyon kanal tedavisi ve pulmoner gen tedavisi gibi semptomatik tedaviden ziyade hastalığı tedavi etmeye yönelik yeni stratejiler geliştirilmiştir. Tedavi seçeneklerindeki önemli ilerlemeler sayesinde, kistik fibrozis, artık pediatrik bir hastalıktan ziyade yetişkin hastalığı haline gelmiştir. Pulmoner gen tedavisi, mutasyon tipinden bağımsız olması ve tüm kistik fibrozis hastalarına uygulanabilirliği nedeniyle özellikle dikkat çekmiştir. Tedavideki en büyük sorun, kistik fibrozis hastalarındaki genetik heterojenite ve karmaşıklık sebebiyle ilaç cevabını öngörememektir. 3D hücre kültürü sistemlerindeki ilerlemeler, rektal hücre biyopsilerinden "organoidler" adı verilen kişiye özel mini organlar üreterek hastalığın modellenmesini ve bireysel ilaç cevabını in vitro olarak tahmin etmeyi mümkün kılmıştır. Bu derlemede, kistik fibrozis tedavisindeki yeni terapötik yaklaşımların, devam eden klinik çalışmaların ve kişiselleştirilmiş tedavi konseptindeki ilerlemelerin özetlenmesi amaçlanmıştır.
Anahtar Kelimeler: : Kistik fibrozis, gen terapisi, gen modülatörleri, rektal organoidler

ABSTRACT
Cystic Fibrosis (CF) is a hereditary, multisystemic disease caused by different mutations in the CFTR gene encoding Cystic Fibrosis Transmebrane Conductance Regulator (CFTR). Cystic fibrosis is characterized by mainly pulmonary dysfunction as a result of deterioration in the mucociliary clearance and anion transport of airways. Mortality is mostly caused by bronchiectasis, bronchioles obstruction and progressive respiratory dysfunction at the early age of life. Over the last decade, new therapeutic strategies rather than symptomatic treatment have been proposed, such as small molecule approach, ion channel therapy and pulmonary gene therapy. Due to considerable progress in the treatment options, cystic fibrosis has become an adult disease rather than a pediatric disease in recent years. Pulmonary gene therapy has gained special attention due to its mutation type independent aspect, therefore

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applicable to all cystic fibrosis patients. Major obstacle is to predict the drug response of cystic fibrosis patients due to genetic complexity and heterogeneity. The advancement of 3D culture systems has made it possible to extrapolate the disease modelling and individual drug response in vitro by producing mini adult organ that have been termed “organoids’ from the rectal cell biopsies. In this review, we aimed to summarize the progress in the novel therapeutic approaches, ongoing clinical trials, and precision medicine concept for cystic fibrosis.
Keywords: Cystic fibrosis, gene therapy, gene modulators, rectal organoids

SUMMMARY

Cystic fibrosis (CF) is a hereditary, multisystemic disease caused by different mutations in the *CFTR* gene encoding Cystic fibrosis Transmebrane Conductance Regulator (CFTR). Cystic fibrosis is characterized by mainly pulmonary dysfunction as a result of deterioration in the mucociliary clearance and anion transport of airways. Mortality is mostly caused by bronchiectasis, bronchioles obstruction and progressive respiratory dysfunction at the early age of life. Over the last decade, new therapeutic strategies rather than symptomatic treatment have been proposed, such as small molecule approach, ion channel therapy and pulmonary gene therapy. Due to considerable progress in the treatment options, cystic fibrosis has become an adult disease rather than a pediatric disease in recent years. Pulmonary gene therapy has gained special attention due to its mutation type independent aspect, therefore applicable to all cystic fibrosis patients. Major obstacle is to predict the drug response of cystic fibrosis patients due to genetic complexity and heterogeneity. The advancement of 3D culture systems has made it enable to extrapolate the disease modelling and individual drug response in vitro by producing mini adult organ that have been called “organoids’ obtained from the rectal cell biopsies. In this review, we intended to summarize the advances in the novel therapeutic approaches, clinicalinterventions, and precision medicine concept for cystic fibrosis.
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INTRODUCTION

Cystic fibrosis (CF) is a hereditary, multifactorial, multisystemic disease characterized by obstruction of airways, microbial infection, digestive disorders and other complications. CF is known as the most common autosomal recessive disease of the Caucasians.[1]

Although the incidence of disease varies greatly throughout worldwide; the highest incidence rate is seen in Northern Europeand United States with1/3000, 1 / 4000-10000 in Hispanics, 1 / 15.000-20.000 in Afro Americans. In Turkey, the incidence rate was known as 1/3400, close to the regions with the highest incidence rate. Globally, around 70,000 to 100,000 people suffer from CF.[2]

Cystic fibrosis is caused by different mutations in the *CFTR* gene encoding Cystic fibrosis Transmebrane Conductance Regulator (CFTR) which regulates the mucociliary clearance and anion transport in airways.[3] The *CFTR* gene is located on the long arm of the chromosome 7 and CFTR protein product consist of 1480 amino acids in length. CFTR acts as a cAMP regulated chlorine channel in apical membranes, providing Na^+ and water transport from epithelial cells in many organs and glands.[4] CFTR dysfunction primarily affects epithelial cells and causes to chronic microbial infection, and subsequently airway inflammation. Mortality from CF is commonly caused by bronchiectasis, bronchioles obstruction and progressive respiratory dysfunction.[5] The severity of the disease is directly proportional to what extent the lungs are affected and varies by person.[6]

The pathophysiology of CF can not be explained by a single hypothesis. The most common theory is the excessive reabsorption of Na^+ and water from the airway surface, resulting a more viscous and elastic state of the airway secretions. These changes in the secretions cause dehydration of airway surface and the formation of mucus plugs; mucociliary clearance becomes insistent. In addition to these changes, low HCO_3^- further affects the microenvironment by converting pH to more acidic. Since the bacterial eradication in the airways is pH dependent, changes in pH disrupts the natural immunity by attenuating the effectiveness of endogenous peptides.[7-9] In addition to these changes, decreased HCO_3^- levels contribute to the increase of mucus intensity.[10] This leads to accumulation of secretions and obstruction of the airways that starting from bronchioles. Mucociliary clearance of inhaled microorganisms that trapped in mucus becomes gradually difficult.[11] In a typical CF infant, *Haemophilus influenzae*, *Staphylococcus aureus*, or both rapidly colonize and *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Burkholderia cepacia* may all comprise even in infants.[12] In a short time, *P.aeruginosa* becomes the most dominant microorganism in the airways. *P. aeruginosa* is the main pathogen in the CF patients and its prevalence is around 70% in CF adults.[13] *P.aeruginosa* forms a polysaccharide film which protects itself to antimicrobial agents. Therefore, bacterial binding to the epithelial cells increases and bacterial clearance decreases with natural immune mechanisms.[14, 15] The management of pulmonary

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infection has a great significance since it affects the time of survival.[16] The most important concern for the CF treatment is the increasing bacterial resistance to standard antibiotics. CF also affects various organs and systems such as the intestinal tract, biliary tract, pancreas, genitourinary system. Co-morbidities are pancreatic malabsorption (malnutrition), biliary cirrhosis and infertility. Pancreatic and bile duct epithelial cellsare affected by CFTR dysfunction as well. Chronic obstructive pancreatitis is observed due to excessive mucus secretion. Severe pancreatic exocrine deficiency causes symptomatic fat malabsorption.[17] If the pancreatic insufficiency can not be controlled, this may cause damage toislet cells and leads to insulin deficiency and CF related-diabetes mellitus (CF-DM). The vascular outcomes of diabetes is evidential in typical DM patients, however in CF-DM patients, nutritional and pulmonary outcomes might be life-threatening. The first treatment option is insulin (i.m) rather than oral antidiabetics in CF-DM patients after the endocrinologic consultation, unlike the typical type-2 DM patients.[18] The intravenous (i.v) administration of aminoglycoside and CF-DM are the major causes of renal failure in cystic fibrosis patients.[19]

The main objective of treatment of CF is to remove excessive mucus from the lungs, to control pulmonary infection, improve pancreatic insufficiency and malnutrition. This perspective has lead to a significant increase in the life time and quality of CF patients in recent years. In this review, we aimed to summarize the novel treatment options and innovative therapeutic approaches for CF.

Classification of CFTR Mutations

To date, approximately 2000 different types of mutations have been identified in *CFTR* gene.[20] However 15% of those are not associated with CF.[21] The most common mutation, called ΔF508, is the 3 base deletion leading to loss of phenylalanine at position 508 in the CFTR protein.[22] The ΔF508 mutation accounts for two-thirds of all the CF alleles.[23] Approximately 90% of CF patients carry at least one copy of the ΔF508 mutation.[24] Determination of the CFTR mutation type has a great importance, since the mutation type appoints the disease phenotype and draws the way of the treatment strategy. Cystic fibrosis is classified according to the step in which the mutation takes place. The conventional classification system divides CFTR mutations into 6 categoriesaccording to CFTR synthesis, trafficking or function. However De Boeck and Amaral[20] grouped mutations into seven classes according to functional defects and separated the previous class I mutations into class I (stop-codon mutations) and a new class VII (no mRNA transcription) (**Table-I**). Classification of mutations helps our understanding of the CFTR defect, however mutations might be more than feature.[25] Class I, II and III mutations are related with no CFTR function and severe phenotype. However class IV, V, VI and VII mutations have residual functional CFTR protein and therefore moderate phenotype and pancreatic insufficiency.[5]

Mutations of class I include nonsense, frameshift, or mRNA splicing mutations leading to absence of CFTR expression, therefore resulting with reduced the number of CFTR channel. Class II mutations, including ΔF508, lead to faulty CFTR processing. Even if CFTR is properly synthesized, missense and in-frame deletion mutations interrupt CFTR folding and trafficking. Some Class II mutations partially disrupt protein stability. In Class III mutations, channel gating is defective due to diminished ATP binding to the channel and result with impaired chloride transport. In class IV mutations, chloride transport is disrupted due to the abnormal CFTR channel pore. Class IV mutations often result with a milder phenotype because of the partial CFTR function. Low amount of CFTR protein is available, however aberrant splicing defects lead to defective mRNA processing (no full lenght or stable mRNA). Class VI mutations are characterized by a functional but unstable CFTR protein, premature degradation of CFTR results with the high CFTR turnover at the cell surface. The last category, class VII mutations consist of large deletions on *CFTR* gene and therefore no mRNA transcription process.[20, 21, 26, 27]

New Treatment Approaches

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New Options in the Management of Pulmonary Infection

Ceftazidime/avibactam is a new cephalosporin-beta lactamase inhibitor combination which is effective for the multiple drug resistant infections.[28] Although ceftazidime has been in clinical use over many years as an antipseudomonal, its efficacy is uncertain due to decreased sensitivity in recent years. Ceftazidime/avibactam combination offers a potential improvement for the CF pulmonary infections involving *P. aeruginosa.*[29, 30] Combination with avibactam increases the activity of ceftazidime against *Enterobacteriaceae* and *P. aeruginosa*, since avibactam inhibits serine β-lactamases including ESBL, AmpC and KPC. On the other hand, avibactam does not increase ceftazidime activity against *Acinetobacter spp*., *Burkholderia spp*. or most of anaerobic gram (-) bacillus.[31] Co-administration of ceftazidime/avibactam and aztreonam showed succesful results for Extremely Drug Resistant (XDR) *Burkholderia multivorans* infections.[32]

Ceftolozane/tazobactam is a novel β-lactam/β-lactamase inhibitor combination approved by the Food and Drug Administration (FDA) in 2014 for the treatment of complicated intraabdominal and urinary tract infections.[33] Ceftolozane/tazobactam is promising for the treatment of *P. aeruginosa* infection in CF patients, alone or in combination with tobramycin or amikacin. The efficacy of amikacin and ceftolozane/tazobactam combination is higher than tobramycin-ceftolozane/tazobactam. These encouraging progress lead to further clinical research against Multi Drug Resistant (MDR) *P. aeruginosa* infections in CF patients.[34] Neither ceftazidime/avibactam nor ceftolozane/tazobactam combination has been approved for the patients under the age of 18 yet, but the pediatric population studies are under investigation and currently in Phase II. [35-37]

Inhaled antibiotics are other widely used treatment option alone or in conjuction with oral antibiotics to prevent pulmonary exacerbations. The use of inhaled antibiotics has advantages compared to i.v. administration or oral route. Increased antibiotic concentration at the infection side through the inhalation enhances the bacterial eradication and systemic side effects such as nephrotoxicity and ototoxicity can be avoided.[38]

Inhaled antibiotics for CF are aztreonam lysine, tobramycin inhalation powder/solution, inhaled colistin, liposomal amikacin, liposomal ciprofloxacin, and inhaled levofloxacin.[39-42] Inhaled tobramycin and inhaled aztreonam are the two inhaled antibiotics with FDA approval. Liposomal amikacin has been recently approved by FDA in 2018.[43]

Inhaled colistin (colistimethate sodium) has been approved by European Medicines Agency (EMA), but not FDA yet. Other antibiotics i.e. liposomal ciprofloxacin, and inhaled levofloxacin have not been approved for CF. These are under investigation in earlier stages of development, in phase studies.[44]

Ion Channel Therapies: Non-CFTR modulating therapies

Inhibition of Na^+absorption

Fluid hydration in the airway depends on Cl^--bicarbonate secretion by CFTR channels and sodium absorption mediated by epithelial sodium channels (ENaC). Although the CFTR channel defect mainly affects the secretion of Cl^- and bicarbonate ions from epithelial cells, it actually leads to deterioration in the secretions and absorption of electrolytes.[45] Increased Na^+ absorption (2-3 times folder than normal) is observed through ENaC, as well as impaired Cl^- secretion. Na^+ hyperabsorption leads to more dehydration of respiratory secretions and further deterioration of mucociliary clearance. Blockage of the epithelial Na^+ channel and prevention of Na^+ hyperabsorbion has been recommended as a treatment strategy.[46]

Amiloride, is the first generation of potassium-keeping ENaC antagonist, developed as a sodium channel inhibitor in the 1960s. Although intranasal administration of amiloride has reduced the pulmonary mortality rate, the risk of hyperkalemia limited its use.[47] However, the study had to be terminated due to acute hyperkalemia caused by inhibition of ENaC in the kidneys.[48]

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AZD5634 is a new inhalable, second-generation amiloride derivate and it is well tolerated without considerable hyperkalemia risk.[49] ENaC antagonists QBW 276 and BI 443651 have undergone clinical investigation and demonstrated remarkablesafety profiles in Phase I trials. However, phase II/efficacy outcomes are still pending.[50, 51]

SPX-101 is an another inhalable ENaC inhibitor peptide has undergone Phase II trials.[52] SPX-101 showed positive and significant results without causing hyperkalemia. The aerosol administration of the antisense oligonucleotides may provide an alternative approach.

Stimulation of Cl^- secretion

Luminal Cl^- secretion of epithelial cells is mediated by CFTR and alternative chlorine channels. Increased activity of alternative chlorine channels like Calcium-Activated Chloride Channel (CaCC) in the lower respiratory tract may compansate decreased or absent CFTR function and improve the clinical status of CF patients.[53]

Activation of $P2Y_2$ nucleotide receptor activates the CaCCs by causing a rapid increase in cytosolic free calcium concentration. ATP and UTP, endogenous $P2Y_2$ receptor ligands increases ion and liquid secretion.[54] However short half-lifes of extracellular ATP or UTP limit their clinical utility. To induce chlorine secretion by $P2Y_2$ mediated CaCC pathway, more stabl inhaled $P2Y_2$ receptor agonists were needed to be developed. Denufosol is an inhaled $P2Y_2$ receptor agonist that increases the Cl^- ion and fluid secretion in the luminal clearance by $P2Y_2$ mediated CaCC stimulation. Although denufosol was found to be effective and well-tolerated in mild CF patients[55, 56], it failed in the Phase III step due to unsatisfactory results in terms of the pulmonary function. Another reason of failure is its short half-life.[57]

Moli1901, also known as duramycin, a stable 19-residue-polycyclic peptide which is derived from *Streptomyces cinnamoneum*, interacts with phospholipids and thereby activates alternative chloride channels by elevated intracellular calcium levels.[58] Although Moli 1901 had shown promise as a chloride channel activator, it could not be further developed due to formulation problems.

Osmotic therapy

Airway surface fluid (ASL) is a thin layer of fluid that covers the lumen surface of the airway epithel and maintains mucociliary clearance, ciliary function, and antimicrobial features of the airway, a key regulator of the airway homeostasis.[59] ASL depletion is an significant factor in the pathogenesis of cystic fibrosis, so it has been shown that osmotic water withdrawal to the airway surface may improve the damaged mucociliary transport.[60, 61] The main building block of cystic fibrosis treatment is actually correcting mucociliary clearance, small molecule approach like CFTR modulators are able to do this by correcting dysfunctional CFTR, and approach to targeting ion channels in airway epithelial cells pharmacologically. Hypertonic saline and dry mannitol powder, which directly correct mucociliary transport, produce an osmotic gradient by drawing water from the aquaporins of epithelial cells.[60, 62] Hypertonic saline, usually used as a 7% solution, induces the release of inflammatory mediators such as prostaglandin E2, altering the rheology of the mucus and increasing mucociliary clearance.[63] Mannitol is a nonionic osmotic agent. The larger size of mannitol has disadvantages over hypertonic saline, and it is difficult to accumulate in small airways, however it is easier to administer via metered dose inhaler (compared to nebulizer hypertonic saline).[64]

Small Molecule Approach: CFTR Modulating therapies

CFTR Modulators were described first by Verkmen in 2003. Cystic fibrosis transmembrane conductance regulator (CFTR) modulators are novel therapeutics that correct CFTR protein production, defective CFTR protein itself and/or its intracellular function. CFTR modulators are significat role in CF treatment since they provide fundamentally therapeutic approach rather than symptomatic therapy, by targeting the production or function of CFTR protein.[65-67] The first group, called as CFTR potentiators, increases the function of the expressed CFTR channels and ameliorates Class III or IV defects even CFTR reaches the cell surface but, non-functional. The second group, called as CFTR correctors, is a group of drugs that can act to

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improve the intracellular processing of proteins, thereby provide CFTR proteins to move to the appropriate site on the cell surface. Finally the third group, CFTR production correctors, induce more CFTR protein production.[68]

The first small molecule defined as CFTR potentiator (potential enhancer) is ivacaftor, which was developed as VX-770 at first.[69] Ivacaftor facilitates the transport of chloride by enhancing the channel opening of the CFTR protein on the cell surface. Ivacaftor is approved by FDA for all class III mutations involving G1244E, G1349D, G178R, G551S, G1370D, S1251N, S1255P, S549N, S549R and particularly G551D mutations for the patients over 12 months of age.[70]

Ivacaftor has been shown to improve lung function and nutritional status, diminish the mortality rate associated with lung dysfunction.[71]

In in vitro studies, ivacaftor improves not only Class III mutations, but also some mutant proteins of IV and V classes.[72] A class IV mutation; Arg117His which leads to impairment of CFTR conductivity is seen approximately 3% of patients with cystic fibrosis.[73]

Novel CFTR potentiator drugs are currently in clinical trials. QBW251 is in Phase II stage of a randomized controlled trial involving 153 patients. Other candidates such as GLPG1837, CTP-656 are also in Phase II.[74, 75]

Despite the fact that ivacaftor improves channel opening time and chloride conductivity, it is not effective in patients who are homozygous for ΔF508 mutation. Since the primary problem in ΔF508 mutation is the inaccurate folding of the protein and inability of reaching the cell surface.[76] Therefore, co-administration of potentiators and correctors is recommended for patients with homozygous for ΔF508. It has been showed that the combination of corrective and potentiator therapies has been more effective than single regimens.[77] FDA has approved lumacaftor -ivacaftor combination for patients with homozygous for ΔF508 mutation who are 2 years and older.[78] Lumacaftor known as VX-809 improves the conformational stability of the ΔF508-CFTR thereby enhances the CFTR processing and its transfer to the cell surface.

Tezacaftor (VX-661) enhances the processing and transfer of CFTR proteins, including both normal and mutant (including ΔF508-CFTR), and thus increases the amount of protein reaching the cell surface. Tezacaftor + Ivacaftor combination was approved by FDA in 2018. It is indicated for the treatment of cystic fibrosis (CF) in patients at the age of 12 and older who are homozygous for the ΔF508 mutation.

The combination of tezacaftor/ivacaftor exhibits fewer side effects than the combination of lumacaftor/ivacaftor especially in terms of increased respiratory symptoms at the beginning of treatment. However there is no therapeutic advantage of tezacaftor/ivacaftor when compared to lumacaftor/ivacaftor combination therapy.

To date, no combination therapy has been approved for patients who have heterozygous ΔF508 mutations (ΔF508 mutation in one alelle + another mutation in other allele= ΔF508-MF) on *CFTR* gene and minimal functional CFTR. Patients who carry two copies of the ΔF508 CFTR mutation (homozygous) are typically treated with a corrective and a potentiator, but not succesfull in heterozygotes.

The new generation CFTR correctors; VX-659 and VX-440 are small molecule drugs that are expected to emerge as part of the triple combination regimen and Phase III studies are in progress.[79]

The VX-659 and VX-440 have a different structure and a different mechanism of action.[80] Thus, the use of two distinct correctors in triple combination therapy acting via different mechanisms has come up. These drugs were developed for use in combination with tezacaftor and ivacaftor (VX-659-tezacaftor-ivacaftor or VX440-tezacaftor-ivacaftor) to return the function of the ΔF508 CFTR protein of patients who have heterozygous ΔF508 CFTR mutation (ΔF508 - MF genotypes) and minimal CFTR function or homozygous ΔF508 mutation. Undoubtedly, the most important outcome of triple combination therapy is the

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treatment success for the heterozygous ΔF508 mutation for which CFTR modulator treatment is not available currently.[81]

Ataluren (PTC124): Potential treatment for Class I mutations

Stop codon mutations account for 10-12% of the all CFTR mutations.[80] This mutation truncates the CFTR protein production by introducing a premature stop in the messenger RNA (mRNA) and leads to unfinished protein formation. Ataluren is novel oral drug that allows ribosomal reading of premature stop codons selectively. Ataluren activity for nonsense mutations has been shownin vitro, however its efficiency remains unclear due to inconsistent results in clinical trials.[82-86] The reason maybe the suppression of atalurene activity by aminoglycosides. [85] Ivacaftor may increase the efficacy of Ataluren by activating a specific protein. Recently completed study in Alabama University at Birmingham has aimed to evaluate effectiveness of ivacaftor in ataluren using a patient after one year of treatment.[87]

Personalized Treatment and Pulmonary Gene Therapy

The precision medicine concept, which acts with the notion of ''there is no disease, there is patient'', is defined as the planning of appropriate treatment by taking into account the patient's genetic background. Undoubtedly, gene therapy is one of the cornerstones of precision medicine and it gave direction to cystic fibrosis studies. Human gene therapy aims to alter, manipulate, or change the expression of a gene or the biological properties of living cells for therapeutic use.[88]

Gene therapy involves the correction of defective *CFTR* gene by inserting an extra copy of a non-defective intact *CFTR* gene into the cell, which is called **gene replacement**, or using specially designed enzymes called nucleases which also function as molecular scissors, which is called **gene editing**. The major obstacle for gene replacement/editing is the gene delivery which is hindered by mucociliary barrier.

Gene editing uses the cell's own DNA repair machinery to correct the mutation in the DNA. Hence, specific gene repair system should be designed for each type of mutation. Recently, CRISPR/Cas9 gene editing technology is rising due to its success. The CRISPR/Cas9 gene editing include a "guide" that locates the mutated sequence in the CFTR gene and "scissors" that break the patient's DNA at the site of the mutation. This DNA damage gets the attention of the cell's DNA repair machinery, which will then fix the DNA breakage This continuously corrects the mutation in the cell therefore its great advantage is; the effect is permanent. However, gene editing tools should be designed specifically for each type CFTR mutations. This creates an obstacle since there are so many types of mutations in CF (approximately 2000 mutations). Besides, gene editing tools can break the DNA in the wrong place (off-target) and cause an error which results in new mutations in other genes. This might lead to unintended consequences, such as an increased risk for cancer.[89]

Although recent technological advances in gene editing (homologous recombination, zinc finger nucleases, transcriptional activator-like effector nuclease, CRISPR/Cas9) are promising, this option have been pushed into the background since there are many types of CF mutations and partially insufficient results.[90] However, the repair of a defective gene with CRISPR/Cas9 tool has huge superiorities compared with the gene replacement therapy. First of all, the corrected gene remains under control of its endogenous promoter, therefore engages with life-long expression by the native regulation in the cell. Moreover, gene replacement has the potential the involvement of foreign DNA, thus increasing risk of insertional mutations. CRISPR/Cas9 gene editing technology is still being improved, promising results were obtained in CF tissue and animal models.[91] CF models, generated in 5 animal species (mice, rats, ferrets, pigs and rabbits), clearly reflect the mechanisms of disease pathogenesis and CFTR function.[92] Recently, the sheep model has been proposed due to the similarity of lung anatomy betwwen two species.[93]

Some researchers have focused on the gene replacement therapy for cystic fibrosis, which includes the presentation the non-defective *CFTR* gene (wild-type) into the lung cells. The

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entrance of functional *CFTR DNA* or *RNA* into the nucleus of lung epithelial cells through a vector and providing the expression of the functional *CFTR* gene instead of the mutant one are the main goals of the treatment.[94]

Mutation type is prominent for small molecule approach, but not for gene replacement therapy. Since there is no need to identify the mutation type of the patient, the gene replecament is suitable for all CF patients.[95] Pulmonary gene therapy is important since it is non- symptomatic and mutation agnostic treatment, especially when compared with the other treatment strategies such as the potentiator and corrector regimens which are limited by genotype. With the discovery of the *CFTR* gene in 1989, studies on gene therapy in cystic fibrosis have gained momentum.[24] Initially, viral and nonviral approaches have been developed to deliver the *CFTR* gene (adeno-associated viruses, adenoviruses, plasmids formulated in cationic liposomes, lentiviral and retroviral vectors). However, the lung that has strong intracellular and extracellular barriers to protect itself from foreign particles is a complex and difficult target organ.[95] Since gene transfer vectors can be deactivated by immune system or inflammation products, this complicates pulmonary gene therapy. The vector carrying the gene reaches the cell surface but receptors responsible for its uptake into the cell may be inadequate, which means inefficient gene transfer. Cystic fibrosis is a lifelong disease and the life cycle of airway epithelial cells requires repetitive administration of *CFTR* gene. All of these can account for the challenges of pulmonary gene therapy.[96, 97] In general, viral vectors are more effective than non-viral alternatives. But non-viral vectors are safer, cheaper and easier to produce.[90]

In vitro studies have showed that the expression of the complementary DNA (cDNA) of the whole *CFTR* gene in the cell improves the anion channel activity. The most important question in this respect is that at least how many cells must be corrected in order to benefit therapeutically. Studies showed that at least 6-10% of airway epithelial cells should be able to express functional CFTR for wild-type anion transport.[98] In 1992, with the production of animal models with cystic fibrosis, there was an increase oin the number of gene therapy studies. In parallel with in vitro studies, transduction of up to 5% of the airway cells with the CFTR expressing vector has reached 50% of Cl^- transport levels in non-cystic fibrosis subject in animal models.[99]

Clinical studies for cystic fibrosis gene therapy were first performed in 1993 by viral and non-viral gene transfer agents from the nasal and bronchial epithelium. Adenoviruses were found safe in repetitive applications and did not trigger any immune response in animal experiments.[100] However it caused immune response in clinical studies.[100, 101] Despite vector modifications afterwards (such as the removal of all adenoviral genes in gutless vectors) to reduce immunodeficiency, there is a poor interest for the development of pulmonary gene therapy with adenoviruses.

The other promising application is recombinant adeno-associated viruses. Adeno-associated vectors are DNA based and lack of some viral genes, such as gutless vectors (also called co-dependent vectors) that require assistance from a helper virus for replication. AAV2 is the first serotype to clinically evaluated in CF patients but it has created frustration in repetitive applications due to changes in the lung function.[96]

Lentiviruses are RNA-based vectors belong to *retroviridae* family. Once lentiviruses enter into the cell, it reverse transcribed into DNA and transcribed DNA is integrated into the genome of the host cell. The advantage of genomic integration is to transfer the undamaged *CFTR* gene into the daughter cells after the cell division. Therefore it provides long term expression. Recombinant lentiviral vectors can be modified to enhance their effectiveness by adding new surface proteins. Question marks remain on whether the genomic integration of lentiviral vectors are safe.[90, 96, 102]

The failure of the viral vectors has led the studies towards the development of non-viral alternatives. The main objective for the development of non-viral (synthetic) vectors is to

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minimize the risk of immunogenicity. Non-viral vectors are circular, plasmid DNA (pDNA) molecules which are complexed with a series of cationic lipids and polymers called as 'lipoplexes' and 'polyplexes'.[96] However, non-viral vectors have no specific components required for cell entry. Nevertheless delivery of the pDNA complicated by cationic liposomes to the lung epithelial by the aerosol system resulted in a 25% correction of CFTR ion transport defect.[103]

In a randomized, double-blind, Phase II trial, non-viral gene therapy pGM169/GL67A was administered for 1 year and pre- and post-treatment FEV1% values of 114 patients were calculated. The FEV1 results showed the modest but significant improvement in the lung function compared with placebo.[104]

To date, the presence of bacterial infection in the lungs has been ignored in terms of the efficacy of pulmonary gene therapy. In fact, the presence of infection can greatly affect the success of gene delivery. In recent years, several studies have been focused on developing multi-functional models that will provide both antibacterial effects and gene distribution.[105] This method provides better protection of DNA during the delivery of the gene and better transfection into the bronchial epithelium, as well as it contributes to bacterial eradication in the airways.

Organoids

As cystic fibrosis is genetically heterogeneous disease, currently available treatment options do not cover all CFTR mutations. Many of the known CFTR mutations are associated with a variety of disease expression and this complicates the estimation of individual disease phenotype. Moreover phenotypic variations can be seen even in the patients having the identical CF-mutations. CFTR genotype-based stratification for medication is challenging for many patients with rare CFTR mutations who are not included into clinical trials due to low prevalence of the mutations ('orphan' mutations frequency <0.1%).[106, 107] Due to genetic heterogeneity, there is great variability in drug responses such as ivacaftor, lumacaftor, or their combination among the CF patients, from no clinical benefit to complete recovery. Therefore, there is a urgent need to elucidate the individualistic drug response from patients who have different types of CFTR mutations. In vitro organoid based functional assays have been developed for this purpose. Organoids are useful tool to predict pharmacogenomics of diverse CFTR mutations and particularly CF drug response.

Organoids, also called mini-organs, are organ-specific 3D cell cultures derived from adult organs or pluripotent stem cells, that reflect the features of parental organ where they originated.[108] They are used to study heterogeneous medical conditions such as cystic fibrosis and cancer where genetics can influence disease severity, prognosis and drug efficacy.[109, 110] Organoids can be used to test drug efficacy and the comparison of different combination treatments. Besides, patient-derived organoids represent an important tool of personalized medicine that allow to predict clinical disease phenotype and how a patient will respond to drug (e.g. CFTR modulating drugs), since they have individual's functional expressions of their own genomes.[111, 112] Drug testing in patient-derived stem cells gathered by rectal biopsy offers an opportunity to select appropriate treatment on an individual basis. Scientists have demonstrated that CFTR function can be readily measured in colorectal organoids by a forskolin-induced swelling (FIS) assay.[107, 113, 114] The efficacy of geneticin, ataluren, ivacaftor, lumacaftor in combination therapy has been tested by FIS method in intestinal organoids with rare mutations.[86]

The first study to measure the corelation between in vivo and in vitro drug response in stem cell culture derived from CF patient was performed by Berkers et al.[111] in 2019. They showed a high correlation between in vitro and in vivo effects of CFTR modulating drugs and demonstrate that organoids have an ideal role in CF modelling with cost effective and patient-friendly manner.

Immunotherapy for cystic fibrosis

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Immunotherapy aims to improve how the immune system works. Chronic elevation in TNF-α, IL-6, and IL-8, as well as IL-17, IL-13 and IL-5 levels in CF have shown to be important for the disease exacerbation. IL-17 levels are found to be high in patients with P.aeruginosa infection.[115] IL-17, IL-5 and IL-13 levels are increasing with the disease exacerbation and IL-17 was shown to be negatively correlated with FEV1 results. It is stated that IL-17 increase was similar in CD4 + Th17 cells and lymph nodes.[116, 117] Another study showed that tryptophan metabolism effects the IL-17 levels and RAR-related orphan receptor c (Rorc) expression. Reduction in tryptophan/kynuren metabolism due to defective indoleamine 2,3-dioxygenase (IDO) causes susceptibility to Aspergillus infections and murine CF sensitivity due to type 17 helper T-cell / regulatory T-cell (Th17/Treg) imbalance. This study emphasizes the importance of immunomodulation in CF through Th17-cell activation and IDO agonist.[118] The first clinical trial for immunomodulator therapy in cystic fibrosis is based on anti-pseudomonas aeruginosa IgY. 20 patients with CF were involved in phase II study. "Anti-pseudomonas IgY" was obtained from the chicken eggs vaccinated with Pseudomonas aeruginosa. Preliminary results showed that it takes much longer to get a new infection and treated patients get fewer infections than controls. In addition, patients had no new opportunistic bacterial or fungal infections (*B. Cepacia, S. Maltophilia, A. Xylosoxidans, atypical Mycobacteria, Aspergillus fumigatus)*, antibiotic use was greatly diminished; lung functions and nutritional status were stable.[119]

4.Conclusion

Over the last decade, cystic fibrosis has become one of the most studied hereditary diseases with novel treatment options. As the availability to access new treatments increased, CF patients have increasing life quality and their survival prolonged. In countries, which devotes more financial resources in the health expenditures, CF has become an adult disease rather than a pediatric disease. However, the average life expectancy may still be in the 20s in low-income economies.

Recent advancements led to the paradigm shift from symptomatic therapies to therapeutic approach which targeted mutant *CFTR* gene. First one is to use small molecules that covers the limited number of CF patients and another one is the pulmonary gene therapy that represents important tool for treatment. Despite many efforts, there is still no FDA-approved pulmonary gene therapy for CF. The major obstacle is the immune surveillance mechanisms of the lung, which hinders repeated administration viral vectors.

CF treatment is always lean on the identification of the underlying genetic defect. Although the clinical outcome is mostly similar, CF patients differ from each other in terms of mutation type and disease progress. Thus, mutation-specific treatment and personalized therapy was an achievable goal for CF. CFTR modulators become remarkable step in terms of personalized treatment in cystic fibrosis. The CFTR potentiator ivacaftor and other correctors; lumacaftor and tezacaftor, have been approved by the FDA for different types of mutations, such as homozygous ΔF508 allele in CF. However, treatment gap for the heterozygous ΔF508 allele still remains. New generation CFTR modulators have potential for the heterozygous ΔF508 allele, by improving the CFTR folding and CFTR trafficking. The development of new generation modulator drugs (e.g. triple combinations) offers an alternative for a much larger CF population, including the heterozygous ΔF508 allele.

Significant efforts have been made to improve the treatment of patients with cystic fibrosis using various strategies targeting the underlying genetic defect and its subsequent results. However, identification of CF drug efficacy is challenging because of the great heterogeneity of CFTR mutations, as well as other unknown factors that contribute to individual drug efficacy. The advencement on 3D culture systems made it possible to extrapolate the disease modelling and individual drug response in vitro by producing mini adult organ that have been termed “organoids’. Further studies are needed to confirm the correlation between in vitro organoid based functional assays and in vivo clinical phenotype and drug efficacy.

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Over the twenty years following the cloning of the *CFTR* gene, the gene therapy for CF has evolved in two distinct areas: gene editing and gene replacement. Gene editing and gene replacement have lacking and superior sides compared to each other. The repaired *CFTR* gene by gene editing technologies remains under control of its endogenous promoter, therefore a definitive and long-lasting treatment is guaranteed. However, the huge number of *CFTR* gene mutations is a major obstacle for gene editing tools. On the other hand, the gene replacement requires repeated administration of the wild type *CFTR* gene throughout the lifetime.

The full restoration of CFTR protein functionality was achieved by using CRISPR/Cas9 gene editing technology in cultured intestinal stem cells (organoids) obtained from pediatric CF patients. Ex-vivo repaired *CFTR* gene by CRISPR/Cas9 in cultured organoids can be reinserted into host succesfully, this might be the beginning of new era. In the near future, it may be possible to obtain stem lung cells from CF patients, engineering them with CRISPR/Cas9 to fix the CFTR mutation, and engraft them into patients' lung where stem cells find their suitable microenvironment to reconstruct the patient's airway.

In conclusion, gene therapies will continue to be an important strategy for CF as well a other genetic diseases, and organoid based regenerative medicine designed with gene engineering technologies can provide the enourmous innovation for CF therapy in the next century.

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Table-I: Classification of CFTR Mutations [20, 21]

Mutation Class	Defect	Phenotype	Example	Treatment Strategy
I	Reduced CFTR protein expression	no protein	Gly542X Trp1282X	Production correctors (Ataluren)
II	Misfolded CFTR protein not transported to the cell surface	no traffic	Phe508del (ΔF508) Asn1303Lys Ala561Glu	Corrector+potentiator (lumacaftor+ivacaftor, VX-661+ ivacaftor)
III	Reduced / lack of CFTR channel opening	impaired gating	Gly551Asp Ser549Arg Gly1349Asp	Potentiator (ivacaftor)
IV	Misshaped CFTR pore restricts Cl^- movement	decreased conductance	Arg117His Arg334Trp Ala455Glu	Potentiator (ivacaftor)
V	Reduced CFTR protein production	less protein	3849+10 kb C→T Ala455Glu 3272-26A → G	No data available
VI	High CFTR protein turnover at the cell surface	less stable	120del23 rPhe508del	No data available
VII	No transcription due to large deletions on *CFTR* gene	no mRNA	dele2,3 (21kb) 1717-1G→A	Unrescuable *(By pass therapies?)*

Kb= kilobases

https://clinicaltrials.gov/ct2/show/NCT00633191?term=immunotherapy&cond=Cystic+Fibrosis&draw=2&rank=1.

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