Journal of High School Science

Therapeutic targets and novel advances in the treatment of cystic fibrosis

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Submitted: November 26, 2021, revision1: March 27, 2022
Accepted: April 1, 2022

Abstract

Cystic fibrosis (CF) is an autosomal recessive disorder that causes persistent lung infections due to the buildup of mucus, which limits the ability to breathe over time. It is caused by a mutation in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene, and symptoms of CF can be diagnosed as early as 6 months old and progressively worsen over time. The first cases of cystic fibrosis were identified in 1935 when the disease was fatal in early childhood, though advances in symptomatic treatment and complication prevention have drastically improved outcomes. Novel approaches to correct the underlying molecular mechanisms of CF, including gene therapy, CRISPR based therapies and CFTR-independent therapies, continue to be explored. This review will provide an overview of current and future treatments for cystic fibrosis.

Keywords

Cystic fibrosis, CFTR, CRISPR, mechanism, COPD, antibiotics, mucus, mutations

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Introduction

The CFTR protein is a regulated chloride channel found in mucus and sweat producing cells, which in turn regulates the movement of water and other chloride and sodium channels in epithelial cells (1). In people with CF, mutations in the CFTR gene cause the CFTR protein to become dysfunctional. Defective CFTR results in thick, sticky mucus which can build up and obstruct the lungs, causing infections, bronchitis, shortness of breath, recurrent coughing and loss of lung function (2,3). Mucus also clogs the pancreas and gall bladder, that normally function to secrete enzyme and bile into the small intestine, triggering malnutrition and abnormalities in digestion which can manifest as poor growth, difficulty in bowel movements and weight loss (4). Endocrine systems are not affected by CF, however the exocrine glands, such as sweat glands, are affected; there is loss of extra salt in sweat, which leads to dehydration, fatigue, weakness, and mineral imbalance (5).

At present, there is no cure for CF, however there are a range of treatments to help control the disease. These include breathing techniques and chest percussion to dislodge mucus, exercise, and physiotherapy, in addition to antibiotics to prevent and control persistent lung infections (6). To overcome pancreatic insufficiency, an enzyme replacement therapy containing proteases, lipases and amylases is prescribed (7). Patients require high-calorie and high-fat diets, supplemental vitamins A, D, E and K, and minerals. Persons with CF also have a higher risk of developing comorbidities, including osteoporosis, diabetes, sinus infections and nasal polyps, impaired liver function and fertility complications (8). The average life expectancy for CF patients is 44 years, however this is increasing as new incrementally better treatments become available. Many patients carefully plan their lives around treatments as well as balancing work and social life.

Molecular mechanisms

The CFTR gene encodes a chloride transporter transmembrane ion channel composed of 1480 amino acids. The ion channel consists of two membrane-spanning domains (MSDs), two cytoplasmic nucleotide-binding domains (NBD) and a regulatory domain (R) (9). Mutations in these five domains can cause variations in symptoms from patient to patient, and over 1000 different mutations in the CFTR gene have been linked to cystic fibrosis. Since CF was first identified, over 2500 new mutations have been discovered (10).

CFTR mutations are grouped into six functional classes, termed Class I through Class VI (11). Class I mutations affect protein synthesis, due to premature stop-codon mutations leading to the formation of mRNA which is degraded through nonsense-mediated decay. Class I mutations are thought to account for 10% of CF cases (12). Class II mutations impact protein trafficking and lead to CFTR protein misfolding and subsequent degradation. The common F508del mutation is a class II mutation and leads to misfolding, polyubiquitination and proteasomal degradation of the CFTR protein (13). Class III mutations impact the ion transport function of the CFTR protein, due to loss of gating function of the ion channel, causing it to permanently exist in the closed, non-functional conformation (14).

Class IV mutations decrease chloride ion transport through the pore, causing reduced

chloride conductance and poor regulation of membrane potential. Class V mutations cause reduced expression of CFTR at the plasma membrane due to defective mRNA splicing. Class VI mutations affect the anchoring and thus the stability of CFTR at the plasma membrane, necessitating high turnover and reduced function (15).

Discussion

Genetic counseling

The incidence of heterozygous CFTR mutations is approximately 1 in 25. If both parents are heterozygous carriers of the CFTR mutation, their offspring will have a 25% chance of homozygous CFTR mutation. If either or both parents are heterozygous carriers, genetic counseling should be offered as a service to provide information about testing and interpretation, and to integrate genetic information into diagnosis and treatment. There are a variety of CFTR genetic tests available, which are generally performed as blood tests or amniotic fluid tests, including CFTR variant panels for routine carrier screening and diagnostic testing, targeted familial variant testing to determine the presence of specific variants in close relatives of a carrier, or commercial next-generation sequencing assays (16).

Gene therapies

Numerous scientific breakthroughs in the past decade have vastly accelerated advances in gene transfer and gene replacement.

Gene therapy is a process by which corrected and functioning genetic material is introduced into cells in order to compensate for abnormal, mutated or malfunctional genes. Although the mutant genes are not removed, the presence of the corrected gene allows the production of fully functioning proteins (17). Gene therapy for cystic fibrosis is delivered by inhalation or *via* intravenous (IV) delivery. Due to the cells in the lungs being surrounded by protective membranes which are coated with thick, sticky mucus, replication-defective vectors are being developed to take the corrected, genetically engineered CFTR gene into the cell (18).

A prominent method of gene therapy, termed integrating gene therapy, involves a piece of DNA that contains the correct version of the CFTR gene being inserted into cells. This DNA is designed to fully integrate into the genome, thus becoming a part of the genome and forming fully functional protein to help the cell carry out its functions. Advantages include the treatment being permanent, which reduces the need for repeated visits and treatments. Unfortunately, there are major risks to irreversibly integrating the CFTR gene, such as the risk of the gene being inserted into a critical part of the genome which contains overlapping genetic material, or into an oncogene.

An alternative method is non-integrated gene therapy. This is a process whereby a piece of DNA with the correct copy of the CFTR gene is provided to the cell, but it remains separate from the genome, avoiding any insertional mutagenesis. However, nonintegrating vectors dilute as replication and segregation occur and thus the treatment may only last for several weeks (19). Yet another method of gene therapy is mRNA therapy, which has demonstrated recent success in Covid-19 vaccination (20). RNA therapy provides functional CFTR mRNA for translation into fully functioning proteins. There is no risk of genome disruption, however repeated doses of treatment are required.

Obstacles in gene therapy for CFTR include the heterogeneous nature of the disease, finding suitable vectors and transfecting patients' lung tissue. Gene therapy has been shown to favorably modify lung function in CF patients (16), however only 27 clinical trials have been performed thus far and many of these were of short duration, with a small number of participants. The vectors used in gene therapies, particularly adenoviral vectors, can pose unknown risks such as random mutations and cancer and hence the ethics of gene therapies with regard to risk and benefit need to be carefully considered. Moreover, high-profile cases such as that of Jesse Gelsinger have influenced public risk perception of gene therapies (21).

Nevertheless, 82% of parents with children with cystic fibrosis were of the opinion that gene therapy was the most important research area in cystic fibrosis (22), and 99% of respondents felt it was ethically sound for children to be given the opportunity to be involved in gene therapy trials provided that they were carefully run, with safety issues being the priority.

Stem cell therapies

Cell-based approaches for cystic fibrosis have advanced significantly following the discovery of induced pluripotent stem (iPS) cell approaches.

There are many advantages to iPS technologies, including the avoidance of issues of histocompatibility and the fact that abundant somatic donor cells can be used. However, methods for ensured reproducibility, functional integration and long-term maintenance are still under consideration, as are questions surrounding the safety of engrafted cells (23). Ethical questions regarding iPS include the fact that they have the potential to become embryos if exposed to the correct conditions. A combination approach of iPS and gene editing can be used to re-implant and engraft corrected cells into patients' lungs, however research remains at an early stage.

CRISPR

CRISPR-Cas9 is a precise and efficient gene-editing technology that can be used to modify, delete or correct specific regions of DNA. Discovered by Jennifer Doudna and Emmanuelle Charpentier in 2011, CRISPR (or "clustered regularly interspaced short palindromic repeats") was first discovered in the immune response of E.coli (24). CRISPR-Cas9 gene editing technology involves two essential components, the Cas9 (CRISPR-associated protein 9) enzyme and a guide RNA, or gRNA, to match a desired target gene and specify the location of cleavage by the Cas9 endonuclease. In order for cleavage to occur, a specific sequence of DNA consisting of 2-5 nucleotides must be at the 3' end of the guide RNA; this is termed the protospacer adjacent motif (PAM). To enable precise gene editing, homology-directed repair is used to precisely repair the cut, using a homologous piece of DNA delivered with the Cas9 and gRNA system as a template for repair (25).

In CF treatment, CRISPR-Cas9 has been shown to correct F508del, the most common mutation in CFTR, in patient-derived organoids (26). CFTR channel function was restored in this experimental system. Moreover, CRISPR-Cas9 has been used to generate new animal models

of CF which will be helpful in testing and validating future therapies.

Despite its revolutionary nature, many ethical questions have been posed around regulation of CRISPR-Cas9 and concerns around the generation of 'designer babies'. Human germline genome editing has been attempted by Jiankui (27) which led to worldwide concern around the ethics and societal debate around heritable genome editing interventions. International commissions have been set up to detail scientific and ethical concerns and to define standards for evaluating whether CRISPR-Cas9 mediated germline editing should proceed.

Genetic engineering can also be used to address secondary causes of symptoms in CF patients. For instance, pulmonary secretions and sputum in CF patients contain significant concentrations of DNA released by leukocytes which accumulate upon infection, and this DNA contributes to the highly viscous nature of the pulmonary secretions. Medication such as Pulmozyme®, a recombinant human deoxyribonuclease I (DNAse), helps to cleave DNA and reduces the viscoelasticity of sputum in order to better manage symptoms. The recent discovery that oligodeoxynucleotides with a pathogen associated molecular pattern (PAMP) induce the release of mtDNA from leukocytes as extracellular web-like structures (28) prompts the question that might not a simultaneous release of DNAse be engineered into this cascade? The potential to utilize CRISPR-Cas9 technology to engineer leukocytes to express and secrete site and event specific DNAse instead of; or in conjunction with; extraneously adminstered antibiotics is not entirely unlike the introduction of the highly effective immune-checkpoint inhibitors into the cancer-fighting therapeutic armamentarium.

Recent CFTR drugs

With CRISPR's legacy in a holding pattern until clinical trials are successful and it becomes more accessible to the public, several drug advances have been made in the past year. Vertex's Kaftrio, otherwise known as Trikafta® , is a triple combination therapy, combining Tezacaftor, Ivacaftor and Elexacaftor. It demonstrated a 14% increase in lung function for patients with a single F508del mutation, and a 10% increase in patients with a double F508del mutation (29,30). Elexacaftor and tezacaftor are CFTR modulators, which facilitate processing and trafficking of the CFTR to the cell surface. Ivacaftor acts as a potentiator, enabling the opening of an otherwise dysfunctional chloride channel.

Ivacaftor in combination with Lumacaftor, which acts as a protein-folding chaperone, preventing destruction of misfolded CFTR protein during the protein trafficking process, is another combination therapy which increases the quantity and function of the F508del CFTR protein at the cell membrane.

CFTR-independent approaches

Approximately a tenth of individuals diagnosed with CF have a nonsense mutation that results in very little to no CFTR production and hence CFTR modulators and potentiator therapies are of no use. In addition, therapeutic approaches independent of CFTR are likely to be suitable for all CF patients. There are a number of CFTR-independent treatments available.

Amphotericin B, a small molecule which forms anion channels, can act as a surrogate for the CFTR channel by secreting HCO_3^- to the airway surface liquid, thereby increasing pH, decreasing viscosity of airway mucus and decreasing bacterial activity in airway epithelia in individuals with CF (31), providing a potential future treatment avenue. Indeed, amphotericin B has been shown to increase anion secretion as measured by transepithelial voltage in primary cultures of CF airway epithelia. This is further validated by the use of intranasal Fungizone®, a clinically approved formulation of amphotericin B, in patients with CF who were not using CFTR modulators. Fungizone® demonstrated a statistically significant change in nasal potential difference which is a key clinical biomarker used to evaluate experimental therapies. These results suggest that amphotericin B has the potential to impact physiology, though further clinical studies to assess whether it can improve lung function are necessary.

Another mechanism for enhancing anion secretion via non-CFTR mediated means is potentiation of TMEM16A (32). TMEM16A conducts Cl^- and HCO_3^- across the airway epithelium, much like CFTR, and selective TMEM16A potentiators such as ETX001 have been shown to increase anion secretion and fluid secretion in human bronchial epithelial (HBE) cells derived from CF patients.

Targeting the epithelial sodium channel (ENaC) with ENaC blockers offers another approach to increase airway fluid volume available to rehydrate mucus and therefore increase mucus clearance (33). Initial Phase 1 trials of an ENaC ion channel inhibitor, ETD001, are underway (34). Inhaled ENaC antisense inhibition reduces ENaC mRNA levels and thus ENaC expression; a recent trial of IONIS-ENAC-2.5Rx demonstrated significant decreases (55.6%) in ENaC expression in healthy individuals (35).

Defective mucociliary clearance is a key symptom of cystic fibrosis which can lead to severe infection and blockages. Current strategies to relieve mucolytic blockages include high-frequency chest oscillation vests. Another novel approach is a nebulized product comprised of bicarbonate, glutathione and ascorbic acid called ARINA-1 (36). In particular, the combination of bicarbonate and glutathione has been shown to increase mucociliary transport rate in human primary bronchial epithelial cells with a F508del double mutation, whilst ascorbic acid exerts antioxidant properties to reduce inflammation.

Many CF patients suffer from chronic lung infections caused by P. aeruginosa species, which are particularly pathogenic and resistant to antimicrobials (37). This is due to the ability of P. aeruginosa to form biofilms, which are an assemblage of bacteria surrounded by a thick, protective matrix of exopolymeric substances (EPS) such as proteins, polysaccharides, lipids and DNA, which protect bacteria from the effects of antibiotics. Biofilm infections have historically been treated with high doses of antibiotics in combination or through surgical removal of the biofilm, however a novel approach is to combine the traditional use of antibiotics such as tobramycin with glycoside hydrolases which degrade the glycosidic bonds of polysaccharides and thus break up the biofilm (38). The glycoside hydrolase PslG degrades Psl, the most dominant polysaccharide in P. aeruginosa biofilms, and PsIG treatment in combination with tobramycin could therefore

be a useful and non-surgical two-pronged approach against CF P. aeruginosa biofilm infections. In C. elegans infected with P. aeruginosa, using lipid liquid crystal nanoparticles (LCNPs) to co-deliver PslG and tobramycin improved their antimicrobial effect by at least 10-fold compared to the unformulated combination (39), suggesting that the use of LCNPs as a delivery system may have promise in CF patients.

An alternative approach to overcome bacterial biofilms is to alter intracellular signaling mechanisms needed to maintain biofilms. Bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) is a second messenger required for regulating key biofilm genes (40), and decreasing cyclic-di-GMP level has been shown to disperse P. aeruginosa biofilms in mice (41). Nitric oxide (NO) increases concentrations of phosphodiesterases which reduces the levels of c-di-GMP and thus in turn helps disperse biofilms (42). A donor NO compound, spermine NONOate, which releases NO locally, has been shown to reduce biofilm biomass of P. aeruginosa clinical isolates from CF patients (43). Another option is inhaled NO, which is currently being tested in a phase II clinical study (44). The Cystic Fibrosis Foundation has recently awarded $2.17 million to Beyond Air® to support the development of a portable inhaled nitric oxide treatment (45). A further approach is increasing endogenous NO production through inhibition of arginase, which competes with nitric oxide synthase (NOS) for L-arginine as a substrate. An arginase inhibitor CB-280 is currently in a phase 1B trial (46).

Often, antibiotics fail to completely eradicate P. aeruginosa infections, leaving behind antibiotic-tolerant 'persisters' which pose a threat of reinfection and treatment failure. A novel approach to eliminate P. aeruginosa persisters in CF patients is *via* the use of large molecule polycationic glycopolymers such as poly (acetyl, arginyl) glucosamine (PAAG) which rapidly permeabilize Gram negative bacterial membranes. PAAG has been shown to completely eliminate P. aeruginosa persister cells in vitro within 24 hours of treatment (47). In addition, PAAG has been shown to have antibacterial activity against methicillin-resistant Staphylococcus aureas (MRSA) (48) indicating promise of this technique in another multidrug-resistant bacterial strain. PAAG has also been shown to sensitize multidrug-resistant strains of Burkholderia cepacia isolates from CF patients to tobramycin and meropenem antibiotics (49). Combining recent insights, a novel treatment strategy might involve using LCNPs to co-deliver PslG, tobramycin and PAAG to potentiate antibacterial sensitivity.

CFTR transports a number of anion species, including thiocynatate, $[SCN]^-$ which is important to avoid the accumulation of harmful hypochlorite, $[OCl]^-$. In human epithelial cell lines, overproduction of hypochlorite has been shown to cause severe cell injury and death (50). Nebulized thiocyanate decreases inflammation in P. aeruginosa lung infection mouse models (51). Using the thiocyanate or an analog such as selenocyanate may improve resolution of lung inflammation and ability to clear bacteria from the lungs in cystic fibrosis.

Conclusions

The treatment of CF in the near future may be expected to shift toward curative – rather than palliative methods. This pivot has as much to do with the emergence of promising new

technological advances, such as CRISPR, mRNA, genetic engineering and the discovery of new pharmacological targets and signaling pathways; as it does with the decrease in the quality of life due to the constant administration of antibiotics as well as the development of resistance to those antibiotics. Particularly interesting are the CFTR mutation independent druggable targets such as EnaC, TMEM16A, arginine and synaptotagmin-1 (52) because modulation of these proteins has the potential to treat the entire CF population; not just the subset with CFTR mutations. This increases the addressable market for the drug; which provides incentive for commercialization.

In the near term, new antibiotics or their combinations, bactericidal drugs such as PAAG, biofilm disrupters such as PsIG or more effective formulations such as LCNPs' will continue to be used. New initiatives to address antimicrobial resistance may yield more effective antibiotics that require a lower dose and have less off-target effects. Bacteriophage based therapies – including CRISPR modified bacteriophages – can also be utilized as bactericidal drugs (53).

Since animal models do not recapitulate either the physiological effects or the pathological features of human CF, microfluidic cell culture devices such as the Lung-on-a-chip (54) are expected to provide accelerated impetus for uncovering more biological pathways that can be exploited as drug targets. These *in vitro* organ level synthetic biology chips will assume increasing importance for decreasing the research-to-clinic time of new drugs.

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