Bench to bedside: Genetic Modifications in Cystic Fibrosis

Rivcka Madhani and Arianna Broad

Abstract: This sentence introduces CF, what it is genetically but also physiologically. This sentence I would summarize what are the current treatments for CF. Our understanding of CF increases as we learn more about the genetic and epigenetic factors that interplay to contribute to pathogenesis. Currently, epigenetic modifications and RNA interference are the most strongly linked mechanisms to CF pathogenesis. In this review, the goal is to critically review the current progress in molecular therapeutics that are being developed for future CF treatment.

Introduction

Cystic Fibrosis (CF) is a recessive autosomal genetic condition. It is caused by a defect in the cystic fibrosis transmembrane regulator (CFTR) gene on chromosome 7 [Dechecchi]. The CFTR protein plays a crucial role in regulating the transport of chloride ions. The most common genetic mutation in the CFTR gene is the deletion of the amino acid phenylalanine at position 508 of the polypeptide chain. The defects in the CFTR gene cause difficulties and complications in the regulation of hydration, volume and pH of mucus at the surface of epithelial cells in the airway tracts in the lungs and in the gastrointestinal tract. The viscous mucus builds up in the airway tracts. This compromises the immune response to clearing bacteria that enter the airways. The excessive unremoved mucus promotes an increase in growth of bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* causing repeated infections. Lung function efficiency significantly falls causing further respiratory conditions. [Dechecchi]. The thick mucus produced prevents digestive enzymes from entering the intestinal tract preventing efficient digestion and

absorption. This results in malnutrition, maldigestion as well as nutritional deficiencies. [srinipong]. These symptoms can also be brought about due to epigenetic reasons. Factors such as DNA methylation, histone acetylation, histone methylation are hypothesized to play a role in the development of CF. Researchers today cannot confirm the underlying reasons for the development and pathogenesis for CF. In this review we are going to understand the causes of CF - both genetic and epigenetic and review treatments developed based on said research. [srinipong].

Background

Ribonucleic Acids (RNA) are nucleic acids that consist of single stranded lengths of information that is genetically coded[1]. They are structurally similar to DNA but have few important differences. There are many different types of RNAs. The focus of this paper will be on non-coding RNAs (ncRNAs). ncRNAs do not code for amino acids and protein structures like other RNAs. They can regulate the expression of genes, physiological processes, developmental mechanisms, and progress of a disease. (Zhang) ncRNAs are further classified into four overarching branches: two of these are long noncoding RNA (lncRNA) or small non coding RNA (sncRNA). lncRNA are longer than 200nt (Varilh) (what is nt?). They regulate DNA sequences and regulate the expression of surrounding genes (Zhang). sncRNAs are shorter than 200nt. One type of sncRNA, microRNAs (miRNAs), have been heavily associated with the development of CF. (Zhang) miRNA are the most widely found ncRNAs. They regulate gene expression within the cytoplasm and nucleus. Furthermore, miRNA also can silence some genes post-transcriptionally (Zhang).

Genetics are not the only contributing factor to CF. The relationship between the genetic sequences and environmental triggers is known as epigenetic mechanisms. There are

[1] https://www.genome.gov/genetics-glossary/RNA-Ribonucleic-Acid

different types of epigenetic mechanisms that can occur. They can be triggered by environmental factors such as food, pollution, and stress. CF is hypothesized to be regulated by epigenetic modifications such as DNA methylation and histone methylation/acetylation. Epigenetic modifications are reversible and can be artificially altered using enzymes. This makes them attractive for future developments of treatments. *(Sirinupong N 2015)*

In DNA methylation, the molecular structure is chemically. A methyl group is added to the DNA through a reversible mechanism catalyzed by DNA methyltransferases (dnmt). There are 3 types of dnmts. They can identify demethylated strands of DNA. The methyl group is added to the carbon at the 5' end of a cytosine *(Moore L 2013).* Majority of DNA methylation takes place on cytosines preceding a guanine base (CpG), which are known to be the most significant type of DNA methylation for gene repression(citation). A methylated cytosine can inhibit transcription by preventing the transcription factors from binding or by encouraging the binding of transcriptional repressors. However, the methylation of non CpG sequences are important for gene regulation in embryonic stem cells. DNA methylation as a trait can be passed from cell to cell via base pairing, allowing reciprocal maintenance of methylation. *(Loscalzo J 2014)*

Histones are the primary DNA organization proteins, by wrapping coiled DNA around themselves to form nucleosome, then subsequently chromosomes[2]. Histones are known to play a key role in regulation of genes. Histones can be modified through acetylation, methylation, phosphorylation, and ubiquitination. CF is regulated through histone acetylation and methylation. *(Loscalzo J 2014)* Histone acetylation targets lysine residues on the histone. It uses primarily uses histone acetyltransferases, but also requires transcriptional factors that assist in the acetylation process on different sites designated for acetylation

[2] https://www.genome.gov/genetics-glossary/histone

within the globular histone core. Histone acetylation is a highly dynamic process that can potentially weaken the interactions between the histone and the DNA by changing the charge of the histone. This modification in charge causes an increase in transcription due to DNA being able to uncoil more readily. The deacetylation of histones is associated strongly with CpG methylation that was discussed before. *(Bannister AJ 2011).* Histone methylation can be further organized into subcategories. Histone methylation can target either lysines or arginines, and both can be methylated to different degrees. *(Loscalzo J 2014).* Histone methylation of lysines can occur up to a degree of 3 and arginines up to a degree of 2. Furthermore, it is not clear in the field if histone methylation is strictly a transcriptional repression or activation modification. It seems that the ability of histone methylation to activate or repress transcription is dependent on the gene which is modified, and further exploration into this question should be completed. *(Bannister AJ 2011)*

Non-Coding RNAs

Over the years lncRNAs have been discovered as a major cause of CF. In a study conducted by Balloy et al (year), the lncRNAs in respiratory epithelial cells of CF patients and non-CF patients were compared. They discovered 17 differently expressed lncRNA that appeared to play a role in CTCF binding (Balloy). CTCF is a multifunctional protein that binds to the chromatin and functions as a transcription factor regulating gene expression (Holwerda, 2013). Furthermore, Balloy et al (year) identified the Bladder Cancer Associated Transcript I (BLACAT1) via RNA-seq was downregulated in CF patients. While there is no direct known mechanism between CTCF and CF development, there appears to be an indirect relationship. The next abundantly identified lncRNA was the Maternally Expressed (MEG9) which was heavily down regulated. MEG9's primary involvement is in transcriptional regulation.

One such gene is called BGas. It was found that this gene targets the CFTR locus specifically to interfere with the binding of proteins essential for maintaining the structure of the DNA and chromatin. The BGas functions as a scaffolding and recruitment protein to separate the CFTR locus via association of chromatin binding proteins such as HMGB1. The HMGB1 proteins are known to distort the structure of the DNA by binding to the DNA in way that closes its accessibility to other transcription factors. Consequently, this results in gene repression. Gene repression does not usually follow this mechanism making this process of repression unique to CFTR.

There are many different small ncRNAs. Some of them have been explored in their relationship with CF, but many have not. Small nuclear RNAs have not been explored in CF gene regulation, however there are potential snRNAs that appear to demonstrate potential therapeutic strategies. Small nucleolar RNAS and piwi-interacting RNAs have not been explored or studied in their relationship with CF (glasgow A, 2018). One type of small ncRNA that has a significant impact on the genetic regulation of CF is microRNA. (Varilh J 2015). 92 differently expressed miRNAs have been identified as contributors to CF. Out of these, 56 are downregulated and 36 are upregulated.

miRNA-126 expression is responsible for an innate immune response to pathogens in the lungs. This response is mediated by high expression of Toll-Like-Receptors (TLRs) in the lung epithelial cells. However, it was experimentally identified that in CF patients miRNA-126 is down regulated as a result of TLR repression, thus preventing an immune response to pathogens in the airway. miRNA-155 was found to be upregulated in CF patients, which is an miRNA known to be associated with the negative regulation of cytokines. Furthermore, in CF patients miRNA-17 and miRNA-93 are downregulated, which targets the silencing of neutrophil chemokine IL-8 transcripts, which in turn promotes an inflammatory response in the CF patients' lungs. In non-CF patients, miRNA-17 and mirRNA-93 are found at

significantly higher levels, which leads to the silencing of IL-8 and therefore, a lowered or nonexistent inflammatory response. (Glasgow A, 2018).While the previously discussed miRNAs contribute to CF pathogenesis indirectly by modulating other proteins aside from the CFTR locus, there are also miRNAs that do regulate the CFTR gene. miRNA-101 and miRNA-145 negatively influence the regulation of the CFTR gene. Varilh et al (year), demonstrated that miRNA-101 and miRNA-145 have a direct impact on the stability of the CFTR gene.

miRNA-199a-5p assists in the condition by inhibiting caveolin 1. CAV1 is used to inhibit TLR4 and thus prevent CF macrophages from appearing. (Glasgow)

To summarize, the pathogenesis of CF involves a myriad of different factors including the increased presence of macrophages in the lungs, ….

DNA Methylation

A study conducted by Chen et al (year), investigated DNA methylation in macrophages that compose bronchoalveolar lavage (BAL) cells in both healthy subjects and CF patients. They identified 109 methylated genes, each with a different methylation pattern. 51 of these showed patterns of excess methylation and 58 showed patterns of less methylation compared to the healthy control subjects. *(Chen Y 2018)* Two of the identified genes that underwent hypermethylation were TNFSF8 and RUNX3. TNFSF8 has been identified to assist in the communication between an innate and adaptive immune response that results in a pro-inflammatory response. Therefore, high methylation levels of the gene causes reduction in expression in the alveolar macrophages and henceforth limiting the communication between the innate and adaptive immune systems. The gene RUNX3 is a transcription factor that regulates chemokines which initiate different leukocyte activity at inflammatory sites. *(Chen Y 2018)*

Three identified genes that underwent hypomethylation were S100A14, LSP1, and OSCAR. The S100A14 gene is responsible for crucial activities that regulate inflammation and thus due to methylation the body's ability to control inflammation in the lung is affected. LSP1 or leukocyte-specific protein 1 is downregulated during hypomethylation as a result of which phagocytic activity of the macrophages is disturbed. OSCAR which is expressed in alveolar macrophages among other cell types is currently thought to promote pro-inflammatory responses of monocytes *(Chen Y 2018)*

$HMOX_1$ is a lung modifier gene that codes for iron homeostasis and cell protection from oxidative damage. It was found to be slightly hyper-methylated. This methylation results in the reduction of the HMOX1 protein which protects cells from varying types of cellular damage, causing CF patients' tissues being exposed to continuous oxidative and ther homeostatic stress. Furthermore, this gene can vary in its methylation state, correlating with the intensity and pulmonary severity in the CF patient. The EDNRA gene is affected the same way. However, the EDNRA gene codes for a protein that causes the contraction of the tracheal smooth muscle. *(Maglhaes M 2017)*

The GSTM3 gene codes for different compounds that are protective and beneficial for CF damaged tissues. Thus, methylation at this gene can impact the severity of the condition as the CF damaged tissues do not receive their normal protection as the gene is repressed. *(Maglhaes M 2017)*

Histone Acetylation

One form of histone modification is acetylation which causes an increase in the expression of the gene acetylated. Histone acetylation is associated with CFTR promoter and intronic enhancer regions. CFTR expressing cells in the gastrointestinal were heavily acetylated in CF patients compared to the non-CFTR expressing cells. The acetylation sites at

intron 1, overlap with the sites of transcription factor binding. The CFTR histone acetylation alongside transcriptional activators, allows an increase in the expression of the CFTR gene. *(Sirinupong N 2015)*

The IL8 promoter gene, when expressed, compounds in CF airway inflammation. The hyperacetylation of the IL8 promoter is implicated in the gene's transcriptional regulation and it is essential for maximal transcription. Consequently, the destructive inflammatory process in the lung is encouraged by IL-8, which in turn promotes CF pathogenicity. (Bartling 2009) It is also important to consider that CF is temporally regulated. Histone acetylation of CFTR associated genes in the fetus changes across the trimesters and the pattern of acetylation is different when compared to adult tissue. (Bergougnoux 2014)

Treatments

There are currently no cures for cystic fibrosis. There are different treatments that can manage the symptoms and increase the longevity of the patient. One approach for developing new treatments is through miRNAs. 2 pathways have been developed based on the different uses of mRNA: (A) miRNA inhibitors called antisense oligonucleotides and (B) miRNA mimics. The miRNA antagonists method is used to suppress genes that are expressed more than they should be. The miRNA binds to the mature miRNA targets which then flag them for degradation, allowing for the normal regulation of gene expression. The main limitation of this therapy is the many side effects that arise. *(Pauline Bardin 2018)* The miRNA mimic approach involves the imitation of endogenous miRNA, in order to reintroduce miRNA that will promote normal gene expression patterns. This is also referred to as miRNA replacement

therapy. The main drawback of this treatment lies in the difficulty of formulating the mimic for its delivery to the target cells.

Other potential treatment methods through gene regulation involve the suppression of BGas gene and the intronic RNA regulatory system which refers to intron 11 embedded in the CFTR gene functions as an enhancer. This would allow CFTR expression to increase as the intronic RNA regulatory system (intron 11 embedded in the CFTR gene functions as an enhancer). S*aayman, S.M. (2016)*

Another approach is to regulate IL-8 production, due to its presence being a strong factor in the development of CF. Strategies to control and regulate the pathogenic elevated levels hold potential for therapeutic uses and new exploration into treatments for CF patients (glasgow)

CF has yet to be thoroughly explored through the lens of epigenetic modifications, however the potential for therapeutics in this sector are abundant. The reversibility of these reactions makes it an attractive source for new treatments. HDAC inhibitors prevent the deacetylation of genes. This acts on the F508del-CFTR gene which improves its expression thus posing as a treatment for CF. DNMT inhibitors increase CFTR transcription during ER stress. While in theory there are many benefits to these treatments, not many clinical trials have been conducted that target epigenetic modifications. *(Sirinupong N 2015)*

Lastly, another treatment for CF being research is gene therapy. This type of therapy focuses on restoring CFTR function by replacing or supplementing the dysfunctional gene with the corrected version of the gene. This type of gene therapy requires the DNA to penetrate the nucleus to be transcribed which is the main issue that arises. Nevertheless, it is currently the most advanced vector system that uses recombinant retroviruses *ex vivo*. *(Wei T 2020)*

Discussion

ncRNA plays a significant role in the regulation of CF. However, while researchers have discovered many genes that could potentially influence the expression of CFTR, the data is still ambiguous. Moreover, the direct mechanism of CF pathogenesis still has uncertainty which is essential to resolve. Nonetheless, different ncRNAs can inhibit or promote the expression of CFTR both directly and indirectly, the primary contributor being miRNA. However, some genes need to be triggered through methylation or acetylation to decrease or increase gene expression respectively. Through this process DNA and histone modifications can change with time and environmental influences; however, some modifications can remain permanent and also be passed down through generations. Each of these contributing factors provide a gateway into tackling CF and forming new and more efficient methods of treating the condition as a whole and not just the symptoms. While all 3 factors (miRNAs, DNA methylation and histone acetylation) appear to be strong areas of investigation for potential treatment, the most promising is for miRNA therapy. Researchers have evidence and plenty of information on the role and influence of different miRNAs in the pathogenesis of CF. This allows the process of developing a treatment more accurate and reliable. Which in essence is why they are a more reliable therapy, due to epigenetic modifications not being fully understood, which may lead to ineffective therapies that have unpredicted side effects.

Conclusion

In conclusion, ncRNAs, particularly miRNAs, have the highest scope of assisting in developing efficient therapies for CF compared to histone acetylation and DNA methylation. However, epigenetic modification still are promising fields for the development of treatments, they require more research into their impacts and how they influence the pathogenesis. It would also be beneficial to fully understand the extent to which ncRNA

influences CF. However, research into these fields today has brought about great insight into managing symptoms and has paved the way for significant developments for achieving an efficient and accurate treatment for CF.

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