

Modifier Genes in Cystic Fibrosis-related Liver Disease

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Abbreviations : ACE, angiotensin I converting enzyme; CF, cystic fibrosis; CFLD, CF-related liver disease; CFTR, cystic fibrosis transmembrane conductance regulator; GSTP1, glutathione S-transferase P1; MBL2, mannose binding lectin 2; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; PAI-1, plasminogen activator inhibitor-1; TGFB1, transforming growth factor beta 1; TIMP-1, tissue inhibitor of metalloproteinase-1, TLR, toll-like receptor.

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Abstract

Purpose of review: Cystic fibrosis (CF; OMIM 219700) is caused by variations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CF-related liver disease (CFLD) affects approximately one third of patients with CF, but the severity of CFLD is highly variable. This review provides the latest knowledge in the pathophysiology and CF genetic modifier research in CFLD.

Recent findings: So far, the only modifier gene validated in CFLD is *SERPINA1* (alpha-1-antitrypsin) Z allele. Recent studies support the view that cholangiopathy arising in CF, is the result of an ill-adapted innate immune response to endotoxins coming from the intestine and triggering a pro-inflammatory response.

Summary: The pathophysiology of liver disease remains uncertain and so far, no therapy has proven effective to prevent the progression of CFLD. A better understanding of the pathophysiology and the effect of environmental and non-*CFTR* genetic influences in the context of CFLD development would help improve management and develop new drug therapies.

Keywords: cystic fibrosis, cholangiopathy, innate immunity, Serpina1

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Introduction

Cystic fibrosis (CF; OMIM 219700) is the most common autosomal recessive disease among Caucasians. CF is caused by variations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Estimates of incidence are of approximately 1 for every 3,000 live births worldwide ¹. The secretory defect in CF, leads to a decrease in the luminal hydration of ducts, physicochemical abnormalities of secretions and duct obstruction. It is a multiorgan disease affecting mostly the lungs, pancreas, sweat glands, and the Wolffian ducts in males. Other clinically relevant manifestations include intestinal obstruction in the neonatal period (meconium ileus) or later in life (distal intestinal obstructive syndrome), and hepatobiliary disease. The median age of survival has increased overtime to exceed 40 years, as a result of neonatal screening and improved management, particularly of lung disease. With the recent development of *CFTR*-directed therapies that correct the function of the defective protein, life expectancy of individuals with CF should continue to increase ².

The clinical phenotype in CF shows considerable variability. Both the number of affected organs and the extent of damage within a specific organ vary substantially between patients with CF. This is particularly true as far as the lung and liver disorders are concerned ^{3,4}.

Liver disease has emerged as a significant contributor to the morbidity and mortality of patients with CF. In 2014, CF-related liver disease (CFLD) was the third most common cause of mortality after cardiorespiratory and transplant-related causes, responsible for approximately 2.8% of deaths ⁵. Focal biliary cirrhosis is the classical histological lesion of CFLD. It has been ascribed to *CFTR* defect in bile duct epithelial cells (i.e, cholangiocytes), which would result in biliary obstruction by inspissated secretions and progressive periportal fibrosis. Focal biliary cirrhosis has been reported in up to 70% in autopsy studies of CF patients ⁶. However, the progression from focal biliary fibrosis to multilobular cirrhosis, which most often manifests as complications of portal hypertension by the end of the second decade of life, occurs in a

minority of patients (5 to 10%)^{4, 7-13}. In the largest series of CF patients with cirrhosis and portal hypertension (561 in total), 90% were diagnosed by 18 years of age with a mean age at diagnosis of 10 years⁸. The progression to cirrhosis and portal hypertension remains unpredictable in the absence of sensitive biomarkers¹³. The other biliary manifestations of CF, including large-duct sclerosing cholangitis, microgallbladder, gallbladder dyskinesia, and symptomatic cholelithiasis have been reported with a wide range of prevalence¹⁴. The factors responsible for this phenotypic variability are not known. Moreover, the pathophysiology of liver disease remains uncertain and so far, no therapy has proven effective to prevent the progression of CFLD^{4, 13, 15}. A full understanding of the pathophysiology and the effect of environmental and non-*CFTR* genetic influences in the context of CFLD development would help improve management and develop new drug therapies.

This review provides the latest knowledge in the pathophysiology of CFLD and CF genetic modifier research in CFLD.

Pathophysiology of CFLD: what is known?

CFTR expression in the hepatobiliary system, is restricted to the apical membrane of cholangiocytes lining the intra- and extrahepatic bile ducts. CFTR drives fluid and bicarbonate secretion in cholangiocytes^{16, 17}. It has been assumed that the importance of CFTR in maintaining homeostasis in the biliary tree was related to the “bicarbonate umbrella” hypothesis, in which a proper alkaline balance is critical to prevent cholangiocyte injury by hydrophobic bile acids^{18, 19}. According to this hypothesis, the loss of CFTR function would contribute to pH dysregulation and thereby would promote cholangiocyte injury¹³. Evidence has also been provided to indicate that the loss of CFTR affected innate immunity and promoted gut dysbiosis, which in turn would induce intestinal inflammation, endotoxemia and CFLD²⁰⁻²⁴. New functions of CFTR in cholangiocytes were recently described, i.e., the regulation of

cell polarity, and of toll-like receptor 4 (TLR4) response *via* the activation of the tyrosine kinase Src ²⁰. This latter study showed that in cholangiocytes, CFTR promoted the assembly of a protein complex that maintained Src in an inactive state ²⁰. Thus, in the biliary epithelium exposed to endotoxins, when CFTR is defective, this complex does not assemble, resulting in the self-activation of Src, and subsequent activation of TLR4 and of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This mechanism may contribute to an increase in the production of proinflammatory cytokines leading to peribiliary inflammation and fibrosis ²⁰. Moreover, the impairment in barrier function is expected to increase the back-diffusion of toxic bile acids, and thereby to further aggravate peribiliary inflammation and fibrogenesis. We also recently showed in CF mouse models, that the development of CFLD features required the combination of CFTR defect with both genetic and environmental factors such as diet-induced dysbiosis ^{24, 25}.

Molecular mechanisms underlying phenotypic differences in CFLD

As an approach to unravel the molecular mechanisms underlying phenotypic differences in CFLD, investigators sought to determine if the presence and/or severity of CFLD was associated with a transcriptomic signature. Pereira et al. provided evidence for a transcriptional basis of CFLD pathogenesis ²⁶. The liver biopsies of children with CFLD compared with those of children with biliary atresia and normal controls displayed differences in the expression of multiple genes related to tissue remodeling and fibrogenesis including chemokines, collagens and matrix metalloproteases. In CFLD, regardless of fibrosis severity, compared with normal controls and biliary atresia, the expression of genes involved in tissue remodeling was decreased, notably the expression of plasminogen activator inhibitor-1 (PAI-1, up to 25-fold) and of tissue inhibitor of metalloproteinase-1 (TIMP-1). By contrast, the expression of genes encoding matrix metalloproteases or involved in the immune response such as interleukins and

their receptors were overexpressed in the liver of patients with CFLD ²⁶. PAI-1 and TIMP-1 are two key regulators of matrix remodeling. PAI-1, encoded by the gene *SERPINE1*, belongs to a family of serine protease inhibitors. It acts as an inhibitor of the tissue-type and urokinase-type plasminogen activators, which convert plasminogen into plasmin ²⁷, a protease required for the activation of matrix metalloproteases and soluble factors such as hepatocyte growth factor or transforming growth factor β . TIMP-1 is an inhibitor of matrix metalloproteases. Therefore, the decrease in the production of PAI-1 and TIMP-1 is expected to increase the activation of matrix metalloproteases and ultimately to increase matrix turnover. Of particular interest, another member of the same family of serine protease inhibitors, namely *SERPINA1*, has been identified as a modifier gene of CFLD ²⁸. Because the genes encoding PAI-1 and CFTR are both located on the long arm of chromosome 7, future studies should also determine whether there is any genetic linkage between these two loci. Overall, transcriptomic analyses supported the role of cytokine and chemokine signaling pathways, hepatic stellate cell activation and matrix remodeling in the pathogenesis of CFLD, as suggested by several studies ²⁹⁻³¹.

We recently demonstrated that CFLD, i.e., cholangiopathy features, was markedly influenced by the genetic background in *Cftr* knockout mice ²⁴. By comparing *Cftr* knockout mice in a congenic C57BL/6J or mixed C57BL/6J;129/Ola background, we found that cholangiopathy occurred only in mice of the congenic background. Transcriptomic analyses of the liver showed that mice in the congenic versus mixed background, overexpressed genes conveying an hyperinflammatory state whereas genes related to innate and adaptive immunity were underexpressed, including the absence of CD1d2 expression, in the C57BL/6J background. Protein-protein interactions predicted by the analysis of genes overexpressed in the liver of the congenic mice formed a large network, displaying links between inflammation, fibrosis and cell (presumably cholangiocyte) proliferation ²⁴. This result supported the view that

cholangiopathy arising in CF, is the result of an ill-adapted innate immune response to endotoxins coming from the intestine and triggering a pro-inflammatory response ²⁰.

Phenotype-genotype correlations

Despite all efforts provided by investigators to elucidate the pathophysiology of CFLD, the reason why only one-third of patients with CF develop liver disease, and why the severity of CFLD is so highly variable remains poorly known.

There are currently more than 2,000 variations listed in the *CFTR* mutation databases (<https://cfr2.org>). Among all *CFTR* variations, the most prevalent one, accounting for approximately 70% of all *CFTR* alleles worldwide, results in the deletion of a phenylalanine at position 508 (F508del). *CFTR* variations are categorized into six classes based on their functional consequences and subsequent residual activity at the apical membrane of epithelial cells. Class I, II, and III mutations (i.e., severe mutations) result in a complete loss of the chloride-channel function; in contrast, class IV, V, and VI mutations (i.e., mild mutations) are associated with altered conductance properties, reduced synthesis or defective stability of normal CFTR, compatible with residual CFTR membrane activity ¹.

CFLD is largely limited to pancreatic insufficient patients with biallelic loss-of-function mutations in *CFTR* (i.e., class I, II, or III mutations on both allele) ^{4, 10, 12, 32, 33}. Meconium ileus was also shown to be a risk factor for severe CF liver disease with portal hypertension ^{4, 7, 10, 12, 34, 35}. There is also an increased prevalence of severe CFLD in males (2:1) over females, in keeping with the fact that male gender is a factor of fibrosis progression in all chronic liver diseases ^{4, 8}.

Recently, we have confirmed in a large cohort of CF patients that the F508del homozygous *CFTR* genotype was a risk factor for the development of liver involvement and severe CFLD ⁴. However, the heterogeneous phenotype of liver involvement in CF patients bearing the same

CFTR genotype, including CF siblings, suggests that non-*CFTR* genetic variations, i.e., modifier genes contribute to the risk of severe CFLD ^{36, 37}, and to the liver phenotypic heterogeneity, as shown for lung and intestinal phenotypes in CF ^{3, 38, 39}. A poor concordance of liver disease in sibling pairs with CF has been reported, which argues against a major role of environmental factors ⁴⁰.

The CF genetic modifier research

Initial searches for non-*CFTR* genetic variants that might influence liver phenotype in patients with CF, used a candidate gene approach, which is based on biological plausibility. However, this approach has not been conclusive so far, mainly because the understanding of the pathophysiology of CF liver disease was insufficient. Genes that have been studied as putative modifiers of CF liver disease include *SERPINA1*, encoding the alpha-1-antitrypsin protein, genes which variants cause hemochromatosis (*HFE*, *TFR2* and *FPN1*), and genes encoding glutathione S-transferase P1 (*GSTP1*), mannose binding lectin 2 (*MBL2*), angiotensin I converting enzyme (*ACE*), transforming growth factor beta 1 (*TGFB1*), interleukin 8 (currently known as *CXCL8*), as well as ATP-binding cassette subfamily B member 4, which is defective in progressive familial intrahepatic cholestasis type 3, intrahepatic cholestasis of pregnancy and drug-induced cholestasis ^{28, 41-44}. However, only *SERPINA1* has been confirmed as a modifier of liver disease severity in CF. Most studies have investigated cohorts of small size and phenotyping did not address the development of severe (biliary) cirrhosis associated with portal hypertension. The largest two-stage control study of CFLD (defined as advanced liver disease with portal hypertension) focused on 9 variations in 5 candidate genes (*SERPINA1*, *ACE*, *GSTP1*, *MBL2*, and *TGFB1*) that had previously been screened in patients with various degree of CFLD severity ^{28, 41, 42, 44}. Genetic analyses of the initial cohort (124 patients with CFLD and 843 control patients without CFLD) showed that a single copy of the *SERPINA1* Z allele and

each additional copy of the *TGFBI* codon 10 C allele were associated with significantly increased odds of CFLD²⁸. In the replication study including an additional 136 patients with CFLD and 1,088 without CFLD, the *SERPINA1* Z allele was confirmed as a modifier of liver disease in CF, but not the *TGFBI* codon 10 variant. Overall, this unique two-stage control study showed a strong association of the Z-allele of the α_1 -anti-protease (*SERPINA1*) gene with CFLD in a total of 260 patients with CFLD versus 1,931 patients with CF and no CFLD. Of particular interest with respect to pathophysiology, the alpha-1-antitrypsin variant protein accumulates not only in hepatocytes but also in cholangiocytes, in subjects bearing the *SERPINA1* Z alleles⁴⁵. One may speculate that the accumulation of alpha-1-antitrypsin variant induces an endoplasmic reticulum stress in cholangiocytes as it does in hepatocytes. This may aggravate endoplasmic reticulum stress induced by the accumulation of F508del CFTR and predispose cholangiocytes to injury of other causes, e.g. endotoxins or toxic bile.

While genome-wide association data have not yet been reported in CFLD, a different methodology was used in a recent work using a system biology approach combining database and literature mining, gene expression study, network analysis, pathway enrichment analysis and protein-protein interactions to examine functional relationships between reported genes⁴⁶. This large study led to the identification of novel modifier genes potentially involved in CFLD: *SLC33A1* (NCBI ID: 9197), *GPNMB* (NCBI ID: 10457), *NCF2* (NCBI ID: 4688), *RASGRP1* (NCBI ID: 10125), *LGALS3* (NCBI ID: 3958) and *PTPN13* (NCBI ID: 5783)⁴⁶. Pathways associated with these genes highlighted the innate immune system and Ubiquitin C (UBC, OMIM: 191340) as the central node linking CF to liver disease^{46,47}.

In conclusion, CFLD is characterized by progressive damage of bile ducts with inflammation, and peribiliary fibrosis in portal tracts. There is increasing evidence to indicate that CFTR

deficiency in the biliary epithelium induces an increased vulnerability to injury of different causes including bacterial endotoxin and a hyperinflammatory response of cholangiocytes or in their vicinity. The reason why, among CF patients with the same *CFTR* genotype, a minority progress to multilobular cirrhosis in the first decade of life still remains poorly understood but genes involved in the innate immune system are candidate modifiers. A multifaceted approach combining genome-wide association studies, and gene expression studies will hopefully reveal significant gene modifiers for CF liver disease. Challenges for the future are to develop novel therapies to prevent or halt the progression of this disease.

Key points

- Cystic fibrosis (CF)-related liver disease (CFLD) manifests as focal biliary fibrosis in approximately one third of patients, but fibrosis may progress to multilobular cirrhosis by the end of the second decade of life in a minority of patients (5 to 10%).
- The heterogeneous severity of liver involvement in patients bearing the same *CFTR* genotype, including CF siblings, suggests that non-*CFTR* genetic variations contribute to the risk of severe liver disease
- So far, the only modifier gene validated in CFLD is *SERPINA1* (alpha-1-antitrypsin) Z allele
- Evidence has recently been provided to indicate that cholangiopathy arising in CF, is the result of an ill-adapted innate immune response to endotoxins coming from the intestine and triggering a pro-inflammatory response
- Using a system biology approach, novel modifier genes potentially involved in CFLD were identified and associated pathways highlighted the innate immune system and Ubiquitin C (UBC, OMIM: 191340), as the central node linking CF to liver disease.

- Genome-wide association data, not yet available, are urgently needed to identify potential targets for modulating liver disease severity in CF.

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