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Small RNA and transcriptome sequencing reveal the role of miR-199a-3p in inflammatory processes in cystic fibrosis airways

Pauline Bardin[1], Emmeline Marchal-Duval[1], Florence Sonneville[1], Sabine Blouquit-Laye[2], Nathalie Rousselet[1], Philippe Le Rouzic[1], Harriet Corvol[1,3], Olivier Tabary[1,*]

[1] Sorbonne Université, UPMC Univ Paris 06, INSERM, Centre de Recherche Saint Antoine (CRSA), Paris, France

[2] Université de Versailles Saint Quentin en Yvelines, UFR des Sciences de la Santé, UMR 1173, Montigny-Le-Bretonneux, France

[3] Hôpital Trousseau, Paediatric Respiratory Department, AP-HP, Paris, France

Correspondence to: Olivier Tabary, INSERM UMR_S 938, Centre de Recherche Saint Antoine (CRSA), Saint Antoine Hospital, 34, rue Crozatier, 75012 Paris, France

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Abstract

Cystic fibrosis (CF) is the most common lethal genetic disease, caused by *CFTR* (cystic fibrosis transmembrane conductance regulator) gene mutations. CF is characterized by an ionic imbalance and thickened mucus, which impair mucociliary clearance, promote bacterial colonization, and the establishment of infection/inflammation cycles. However, the origin of this inflammation remains unclear, although microRNA (miRNA) are suspected to be involved. MiRNA are small non-coding RNA that bind to the 3′-untranslated regions (UTR) of target gene mRNA, thereby repressing their translation and/or inducing their degradation. The goal of this study was to investigate the role of microRNA associated with pulmonary inflammation in CF patients. Through the analysis of all miRNA (miRNome) in human primary air-liquid interface cultures, we demonstrated that miR-199a-3p is the only miRNA downregulated in CF patients compared to controls. Moreover, through RNA sequencing (transcriptome) analysis, we showed that 50% of all deregulated mRNA are linked directly or indirectly to the NF-κB pathway. To identify a specific target, we used bioinformatics analysis to predict whether miR-199a-3p targets the 3′-UTR of *IKBKB* which encodes IKKβ, a major protein in the NF-κB pathway.

Subsequently, we used bronchial explants from CF patients to show that miR-199a-3p expression is downregulated compared to controls and inversely correlated with increases in expression of IKKβ and IL-8. Through functional studies, we showed that miR-199a-3p modulates the expression of *IKBKB* through a direct interaction at its 3′-UTR in bronchial epithelial cells from CF patients. In miR-199a-3p overexpression

experiments, we demonstrated that for CF cells miR-199a-3p reduced IKKβ protein expression, NF-κB activity, and IL-8 secretion. Taken together, our findings show that miR-199a-3p plays a negative regulatory role in the NF-κB signalling pathway and that its low expression in CF patients contributes to chronic pulmonary inflammation.

Keywords: cystic fibrosis; lung; miRNome; transcriptome; miR-199a-3p; NF-κB

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in the Caucasian population [1], caused by mutations in the *CFTR* (Cystic Fibrosis transmembrane Conductance Regulator) gene [2]. *CFTR* encodes a chloride channel [3] essential for the osmotic balance of airway surface liquids. The most common mutation is F508del [4], resulting in the lack of the channel at the apical membranes of epithelial cells and leading to an ionic imbalance and thickened mucus [5]. The resulting hyperviscosity impairs mucociliary clearance, promoting bacterial colonization and the establishment of infection/inflammation cycles which, in the long term, degrade the pulmonary epithelium. Chronic inflammation is the hallmark of CF lung disease with the presence of polynuclear neutrophil invasion and interleukin-8 (IL-8) in the airway of CF patients [6,7] mainly caused by changes in the NF-κB pathway [8,9]. NF-κB is a nuclear transcription factor involved in many cellular processes; it plays a fundamental role in the immune response, especially in the secretion of cytokines, such as IL-8, in the lungs. NF-κB is a dimeric transcription factor present in a latent form in cells and can be induced to bind to DNA at κB sites. The canonical pathway is usually induced by microbial or viral infections and/or pro-inflammatory cytokines, all of which activate the β-subunit of the IκB kinase (IKKβ) complex. IκB kinases (IKK) phosphorylate IκB (inhibitors of κB) bound to NF-κB, resulting in the ubiquitin-dependent degradation of IκB and translocation of NF-κB dimers into the nucleus [10]. In CF, the origin of the deregulation of NF-κB remains unclear, despite extensive publications in the field [11,12].

Here therefore we focused on microRNA (miRNA), as an alternative mechanism that has been suggested to regulate the NF-κB [13-15]. MicroRNA are small endogenous non-coding single-stranded RNA (19–23 nucleotides) that negatively regulate gene expression. The control exerted by a miRNA on its target gene most commonly occurs through the 3′-untranslated region (UTR) of the mRNA, leading to the inhibition of its translation [16]. miRNA regulate more than 60% of human protein-coding genes; hence, they are implicated in normal physiological function [17]. Moreover, miRNA play a critical role in many diseases, in which their expression is deregulated [18-21]. Many studies on CF have focused on the role of miRNA in regulating *CFTR* gene expression [22], but few studies have compared miRNA expression levels between CF patients and non-CF controls. We aimed to understand the role of miRNA in pulmonary physiopathology in CF patients in basal conditions. We assessed miRNA and mRNA expression in bronchial cell specimens from CF patients and non-CF controls, and we also performed an experimental *in vitro* modulation of miRNA expression to elucidate miRNA/mRNA regulation in the context of CF by bioinformatics followed by functional analyses.

Methods

Human bronchial epithelial cell culture

Human bronchial epithelial cells obtained from lung tissues removed during surgery and cultured at an air–liquid interface (ALI) were purchased from Epithelix (Plan-les-Ouates, Switzerland) [23]. Cells were cultured with MucilAir™ culture medium (Epithelix) for at least 8 wk. The permeability of tight junctions and the differentiation were validated by transepithelial electrical resistance measurement (>400 $\Omega.cm^2$) and cilia beating frequency (>7 Hz). Data were generated from human

bronchial epithelial cells from five CF patients homozygous for the F508del mutation and five healthy donors (non-CF). The human bronchial epithelial cell lines CFBE41o$^-$ (CF) and 16HBE14o$^-$ (non-CF) were a gift from Prof. D.C. Gruenert (UCSF, San Francisco, USA). They were cultured in minimum essential medium (MEM) in the presence of Earle's salts and L-glutamine (Thermo Fisher, Villebon-sur-Yvette, France) containing 10% bovine growth serum (Eurobio, les Ulis, France) and 1% penicillin/streptomycin (Thermo Fisher). Cell cultures were grown and maintained at 37 °C in a 5% CO_2 humidified incubator.

Human lung explants and plasma

Human lung explants were collected and processed in compliance with the current French public health legislation (L.1235-2 and L.1245-2 articles, http://www.legifrance.gouv.fr). Each participating institution informed the patients and ensured that they were not opposed to the use of surgical samples, removed during a medical act, for research purposes. Lung fragments were obtained from nine non-CF controls undergoing surgery (45 ± 21 years old) and from eight CF patients (homozygous for the F508del mutation; 35 ± 9 years old) undergoing lung transplantation. For non-CF controls, samples were obtained from a non-pathologic area without inflammatory cells from patients with bronchial carcinoma. After tissue dissection, samples were directly frozen in liquid nitrogen before miRNA/RNA extraction.

Plasma samples were collected during annual blood tests from 26 non-CF controls (30 ± 13 years old) and 25 CF patients (homozygous for the F508del mutation; 15 ± 3

years old). The blood samples were centrifuged for 15 min at 3,000 x *g*. The plasma (supernatant) was harvested, aliquoted, and stored at -80 °C until miRNA extraction.

mRNA and miRNA isolation, purification, library construction, and sequencing

Small RNA (<200 nt) and large RNA (>200 nt) were isolated using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany). RNA purification and library construction were performed as previously described by the transcriptome and epigenome facilities [24]. RNA sequencing (RNA-seq) was performed at the Institut Pasteur (Paris, France). We used 3 µg of large RNA to purify polyadenylated mRNA and to build an RNA library using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA). Ten samples were sequenced on a HiSeq 2000 sequencer (Illumina) in single-end mode to generate approximately 100 million reads of 50 bases per sample. Adapter sequences and low-quality sequences were removed using an in-house program (https://github.com/baj12/clean_ngs). Only sequences at least 25 nt in length were considered for further analysis. TopHat (version 1.4.1.1, default parameters; http://ccb.jhu.edu/software/tophat) was used for alignment against the reference genome (hg19). Genes were counted using HTseq-count.

For miRNome analysis, miRNA-seq was performed at the Institute of Molecular and Cellular Pharmacology (IPMC, Valbonne, France) as described previously [25]. Briefly, small RNA libraries were constructed using a TruSeq small RNA sample preparation kit (Illumina). Small RNA-seq libraries were generated using 200 ng of small RNA as input from each sample. Using multiplexing, the platform combined up to four samples into a single lane to obtain sufficient coverage and ran two technical replicates for each library. T Cluster generation was performed on a Flow Cell v3

(TruSeq SR Cluster Kit v3; Illumina) using cBOT. Sequencing was conducted on the HiSeq1000 platform [24]. The PANTHER (Protein ANalysis THrough Evolutionary Relationships; http://www.pantherdb.org/) classification system was used to classify genes identified in our transcriptomics data according to their molecular functions annotated using ontology terms.

Predicted miRNA target

Several online algorithms (Pictar, TargetScan, miRBase, and miRWalk) were used to identify putative binding sites between the seed regions of miRNA and the mRNA of potential target genes. These algorithms are available online at www.pictar.mdc-berlin.de, www.targetscan.org, www.mirbase.org, and http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/.

Cell transfection

CFBE41o$^-$ cell lines were transfected with miR-199a-3p mimic (5′-ACAGUAGUCUGCACAUUGGUUA-3′) or scramble mimic as the control (Thermo Fisher) using HiPerfect (Qiagen, Les Ulis, France) at 2.5, 10, 30, 50, and 100 nM for 24, 48, and 72 h. After transfection, cells were lysed for miRNA, RNA, or protein extraction.

miRNA and RNA extraction

For miRNA and RNA extraction from human lung explants, 100 mg of pulmonary sample was crushed with a POLYTRON® sonde (PT 3 100, Kinematica, Luzern, Switzerland) in 1 mL of TRIzol® (Life Technologies, Saint-Aubin, France) [26].

For cells, miRNA and RNA were extracted using a Nucleospin miRNA kit (Macherey-Nagel). For plasma samples, miRNA was extracted using a Nucleospin miRNA Plasma kit (Macherey-Nagel). RNA and miRNA were eluted with sterile water. The concentration and quality of the RNA and miRNA were evaluated using a NanoDrop spectrophotometer by Absorbance at 260 nm.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR) analysis

The genes of interest, human miR-199a-3p, *IKBKB*, and *IL8*, as well as the endogenous controls *RNU6B*, miR-103, and *GAPDH*, were reverse transcribed with the TaqMan microRNA and RNA reverse transcription kit using 20 ng miRNA and 100 ng RNA sample. qPCR was performed using a StepOnePlus™ thermocycler (Thermo Fisher) and TaqMan technology. For relative quantification, miRNA and RNA levels were calculated using the $2^{-\Delta C_T}$ method and normalized to the expression levels of *RNU6B*, *miR-103,* and *GAPDH*. Each sample was assessed in triplicate.

Protein extraction and western blot analysis

Proteins were extracted using a lysing solution (anti-protease cocktail, Na_3VO_4, NaPPi). Total protein concentration was evaluated with a Qubit fluorometer (Thermo Fisher). Next, 20 µg of total protein extract was reduced and size-separated on 10% SDS-polyacrylamide gels; transferred to nitrocellulose membranes (Bio-Rad, Marnes-la-Coquette, France); blocked in TBS-T milk; and incubated with specific primary antibodies against IKKβ (#2884), phospho-IκBα (#9246), NF-κB (#6956), and

β-actin (#3700) (Cell Signaling Technology, Boston, MA, USA). The proteins of interest were detected, imaged, and quantified (Chemidoc, Bio-Rad, France).

Luciferase assay

An IKBKB-3′-UTR wild-type plasmid, *CGCCTTGTCTGCACACTGGAGGTCCTCCATT,* (supplementary material, Figure S1) and a mutated plasmid, *CGCCTTGTCTGCTGTGACCAGGTCCTCCATT* were used for the luciferase assay (Tebu-Bio, Le Perray en Yvelines, France). CF cells were seeded in six-well plates and were transfected the next day with 1 µg/ml of the IKBKB 3′-UTR using ExGen 500 (Euromedex, Souffelweyersheim, France). Cells were co-transfected with 30 nM miR-199a-3p mimic or scramble mimic as the control. Supernatants were collected 48 h after co-transfection. For these experiments, we used a Secrete-Pair™ Dual Luminescence Assay kit (Genecopoeia, Tebu-bio), designed to analyse the activities of *Gaussia* luciferase (GLuc) and secreted embryonic alkaline phosphatase (seAP), an endogenous reporter using the same samples from the cell culture medium.

NF-κB activity

Nuclear proteins were extracted from cells transfected with miR-199a-3p mimic or scramble mimic as the control using a Nuclear Extract kit (Active Motif, Rixensart, Belgium). Total protein concentration was evaluated with a Qubit fluorometer (Thermo Fisher). The transcriptional activity of p65 NF-κB in 20 µg of nuclear protein extract from CFBE41o$^-$ cells was assayed using the TransAM^{TM} Transcription Factor Assay kit (Active Motif).

IL-8 assay (ELISA)

The supernatants of cells transfected with miR-199a-3p mimic or scramble mimic as the control were recovered 48 h after transfection. The IL-8 concentration of each sample was determined with the human IL-8 Duo-Set kit (R&D Systems, Lille, France).

Statistical analysis

RNA-seq data counts were analysed using R version 3.0.2 and the Bioconductor package DESeq2 version 1.2.9 (www.bioconductor.org). The generalized linear model included time and condition (CF vs. non-CF). Extracted contrasts included comparisons between the CF and control groups and pairwise comparisons of time points in each condition. Raw *p*-values were adjusted for multiple testing using the Benjamini and Hochberg procedure, and genes with an adjusted *p*-value < 0.001 were considered differentially expressed [24].

For functional analysis, all data are described as mean ± SD. Between-group differences were assessed using the Mann–Whitney *U*-test, Student's *t*-test, and Pearson correlations. Values of $p \leq 0.05$ were considered significant; in the figures, statistically significant differences are indicated for $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Results

miRNome and transcriptome analyses

Differential expression of miRNA and mRNA between CF patients and non-CF controls was assessed by biostatistical analysis. Among the differentially expressed genes, we selected those whose fold change (FC) was ≥2 in upregulation and ≤0.5 in

downregulation. Eleven significantly differentially expressed miRNA were observed when comparing miRNA expression levels between CF and non-CF cells, including 9 upregulated miRNA and 2 downregulated miRNA CF cells. In the same sample, 18 significantly differentially expressed mRNA were observed, including 7 upregulated mRNA and 11 downregulated mRNA in CF cells (Table 1).

We speculated that the integration of both levels of transcript information should considerably enhance the conclusiveness of pathway analysis. Thus, by integrating both the miRNA and mRNA profiles, we aimed to identify specific signalling pathways that account for these regulatory phenotypes; this experimental concept is graphically outlined in Figure 1. To detect miRNA–mRNA networks affecting CF cells, we combined experimentally validated mRNA and miRNA targets, as well as significantly deregulated genes, for pathway analysis using DAVID bioinformatics resources, including BioCarta and the KEGG database (Figure 1). Based on these analyses, we decided to focus our study on miR-199a-3p, a miRNA predicted and validated in other models to control the NF-κB pathway. As many as 50% of the RNA found to be dysregulated in CF by the transcriptomic analysis are associated with this pathway.

Expression of miR-199a-3p*,* IKBKB*, and* IL8 *in CF and non-CF bronchial tissues*

To characterize and confirm the relevance of the chosen pathway in an *ex vivo* model, we first quantified the expression of miR-199a-3p, *IKBKB,* and *IL8* in human lung explants from CF patients and non-CF controls biopsies using RT-qPCR. In the non-CF bronchial tissues, we observed that miR-199a-3p was expressed heterogeneously due to inter-individual phenotypic variability (Figure 2A). In CF patients, miR-199a-3p expression was significantly lower, and *IKBKB* and *IL8* mRNA

levels were elevated compared to those in non-CF controls (Figure 2A–C). According to Pearson's correlation analysis, there was a significant correlation between miR-199a-3p and *IKBKB/IL8* expression in non-CF patients, whereas miR-199a-3p expression was only correlated with *IKBKB* expression in CF patients ($p \leq 0.005$; Table 2). We analysed one miRNA that was unchanged between non-CF and CF cells. We randomly selected miR-23a from the list of the unchanged miRNA. RT-qPCR revealed no significant difference in miR-23a expression between non-CF and CF cells (supplementary material, Figure S2), confirming the small RNA-seq results.

miR-199a-3p *directly regulates IKBKB*

In CF patients, we hypothesized that the expression of miR-199a-3p can modify *IL*8 expression through the NF-κB pathway. To confirm this, CFBE cells were transfected with miR-199a-3p mimic at different concentrations (2.5, 10, 30, 50, or 100 nM; supplementary material, Figure S3) or with scramble mimic as the control for different durations (24, 48, or 72 h; supplementary material, Figure S4). miR-199a-3p expression was analysed by RT-qPCR. The level of miR-199a-3p in the presence of 30 nM of the mimic for 48 h increased by a factor of 300 compared to that in the control. As this represented the optimal conditions, subsequent experiments were performed under these conditions. Next, to test whether miR-199a-3p inhibits *IKBKB* expression by binding to its 3′-UTR, CFBE cells were co-transfected with a luciferase reporter vector containing the 3′-UTR region of wild-type *IKBKB* (WT-IKBKB-3′UTR) or with the 3′-UTR region of mutated *IKBKB* (M-IKBKB-3'UTR) and with miR-199a-3p mimic or scramble mimic as the control. At 48 h, transfection with the mimic induced stronger miR-199a-3p expression than that in the control (Figure 3A). We observed a significant decrease in luciferase activity after the co-transfection of miR-199a-3p

mimic/WT-IKBKB-3'UTR compared to miR-199a-3p mimic/M-IKBKB-3'UTR, and this decrease is more important compared to that of mimic control/WT-IKBKB-3'UTR (Figure 3B). This indicates a direct miR-199a-3p/*IKBKB* interaction at the 3′-UTR region of *IKBKB*.

miR-199a-3p *regulates the NF-κB pathway through IKBKB*

Under the same conditions as described above, we assayed various proteins implicated in the NF-κB pathway (IKKβ, phospho-IκBα, NF-κB, and IL-8). In the presence of miR-199a-3p mimic in CFBE cells, we observed that IKKβ protein expression was lower by 40% than in CFBE transfected with scramble mimic (Figure 4A). Similar results were obtained with other validated miR-199a-3p targets at the transcript level (supplementary material, Figure S5). There appeared also to be a trend for reduced expression (by 14%) of phospho-IκBα in the presence of miR-199a-3p mimic (Figure 4B). Moreover, we observed a 36% decrease of NF-κB protein expression in CFBE cells transfected with miR-199a-3p mimic compared to the control (Figure 4C).

Next, we evaluated the effect of miR-199a-3p on NF-κB activation in the nucleus using the TransAM method. From CFBE cells transfected with miR-199a-3p mimic or control, we extracted nuclear proteins 48 h after transfection. NF-κB activity was measured from these protein extracts through a colorimetric reaction, with the absorbance being proportional to NF-κB activity. We observed that NF-κB activity was significantly lower in CFBE cells transfected with miR-199a-3p mimic (0.2 arbitrary unit (AU)) than that in the control condition (0.6 AU; Figure 4D). Finally, we assayed IL-8 secretion in CFBE supernatants 48 h after transfection with miR-

199a-3p mimic or the control by ELISA. We observed significantly lower IL-8 secretion by cells transfected with miR-199a-3p mimic (2,446 pg/ml) than that in the control condition (3,367 pg/ml; Figure 4E). In contrast, no significant difference in *IL8* mRNA was observed (data not shown).

Expression of miR-199a-3p *in CF and non-CF plasma*

To verify the association between inflammation and miR-199a-3p expression in the plasma of CF patients, we used RT-qPCR to measure the levels of this miRNA in non-CF and CF patient plasma extracts. We found no significant difference in the expression of miR-199a-3p between the plasma of CF and non-CF controls (Figure 5).

Discussion

For the first time, all dysregulated miRNA and mRNA were analysed in the same sample of primary CF bronchial epithelial cells cultured at an air–liquid interface as compared to levels in non-CF cultures, providing a non-biased approach. In this model, we found few dysregulated miRNA and RNA between the primary cells of CF patients and non-CF controls. Interestingly, half of the dysregulated genes were predicted to be associated with the NF-κB pathway, and one miRNA was significantly downregulated in CF patients: miR-199a-3p. Bioinformatics approaches predicted that this miRNA regulates the NF-κB pathway via an interaction with IKKβ. We thus focused our study on miR-199a-3p, and through functional analysis, we confirmed that miR-199a-3p specifically binds directly to the 3′-UTR of *IKBKB* in the CF pulmonary context. Furthermore, we demonstrated that the upregulated expression

of miR-199a-3p leads to decreases in the activation of NF-κB and the secretion of IL-8, the main pro-inflammatory parameter dysregulated in CF airways.

Chen *et al*. [27] demonstrated a functional role for miR-199a-3p in epithelial ovarian cancer cells as a direct inhibitor of IKKβ (*IKBKB*) expression, leading to the modulation of the NF-κB pathway. Dai *et al*. [18] found similar results in endometrial stromal cells, showing through *in vitro* and *in vivo* experiments that NF-κB inhibition through miR-199a-3p transfection could abrogate the development and maintenance of endometriosis, partly by reducing pro-inflammatory and invasion mediators. Our results validate the presence of this regulatory system in lung tissue and provide new insights into the origin of the constitutive inflammation observed in CF patients. If the targets of certain miRNA are tissue-specific, miR-199a-3p can regulate different types of cells by downregulating different target genes [28,29]. However, the regulation of the NF-κB signalling pathway by miR-199a-3p via direct interaction with *IKBKB* appears to be applicable to different cell types (epithelial ovarian cancer cells, endometrial stromal cells, and CF cells) in different pathological contexts, including those involving tissue repair, chemoresistance, tumour progression, and pro-inflammatory environments.

In the absence of inducible inflammation, our results indicate that the NF-κB pathway is constitutively activated in CF cells, leading to the production of pro-inflammatory cytokines such as IL-8, by contrast to the results obtained by Becker *et al*. [30]. Moreover, we demonstrated that different miRNA are dysregulated, and miR-199a-3p can control the NF-κB pathway through IKKβ. Other groups have performed miRNA expression profiling studies and have identified other altered miRNA expression patterns [31,32]. The differences might be explained by the model and/or the method used. For example, Oglesby et al. observed an increase in miR-199a-3p, but CF

bronchial brush sampling might be prone to bacterial infection, confirming the evidence for important roles for miRNA in regulating innate immunity. For the first time, in the context of CF, we demonstrated a new mechanism of inflammation regulation.

To our knowledge, this is the first observation of a possible link between miR-199a-3p expression and IL-8 secretion in bronchial epithelial resting cells. This result provides novel clues among IL-8 expression, and NF-κB activation in the context of CF and highlighting the role of miRNA in this regulation.

The deregulation of inflammatory processes has been shown to pass through the NF-κB and MAPK signalling pathways, notably via p38 and ERK1/2, which are constitutively active in the CF context [33]. However, the causes of the infection/inflammation cycles that degrade the pulmonary epithelium remain unknown. Previous studies in our laboratory showed that mutations in the *CFTR* gene led to chloride dysfunction, resulting in the deregulation of cellular homeostasis and an increase in the intracellular calcium concentration at the beginning of NF-κB hyperactivation [11,34,35]. Nevertheless, there is no direct link between calcium flow variation and NF-κB pathway activation in the literature. According to our results, miR-199a-3p regulates the activation of the NF-κB pathway by targeting the 3′-UTR of *IKBKB*, but the origin of the miR-199a-3p deregulation in CF remains to be identified. Therefore, we analysed the level of pre-miR-199a-1 and pre-miR-199a-2 as a precursor of miR-199a (data not shown) and the level of miR-199a-5p, the other form of miR-199a. We did not observe any significant difference in the pre-miRNA, but we found a significant increase in miR-199a-5p in CF cells (data not shown), as previously demonstrated [36]. We can hypothesise that a post-maturation mechanism could regulate the expression of miR-199a-3p. Even if we cannot prove

and establish this, for the time being, miR-199a-3p could be an intermediary actor between calcium and the NF-κB pathway. Sun *et al*. demonstrated that miR-199a-3p expression is dependent on calcium [37]. Interactions between miRNA and calcium are prevalent in the literature and play fundamental roles in many pathologies [38-40], notably in the cellular processes of proliferation, adhesion, differentiation, and inflammation. For example, miRNA are known to act on genes such as *SERCA*, *IP3R*, and *RYR*, genes that encode calcium carriers located on the endoplasmic reticulum [39]. Thus, one hypothesis is that miR-199a-3p is regulated by calcium in CF. The second hypothesis is that miR-199a-3p is regulated by a pre-existing pro-inflammatory context. The level of miR-199a-3p is decreased in CF cells, leading to increases in the activation of the NF-κB pathway and secretion of IL-8, and this cytokine may act to regulate the expression of miR-199a-3p through a feedback cycle. Further investigations are needed to understand the origin of miR-199a-3p dysregulation in the context of CF.

Based on the lKKβ luminescence assay, the significant decrease (70%) in IKKβ observed in the presence of miR-199a-3p mimic explains the reduction in IL-8 secretion. These relatively low decreases in IL-8 secretion can be explained by the fact that protein translation is under the regulation of other miRNA and not only miR-199a-3p. Sonneville *et al*. [22] reported that more than 49,000 proteins (representing half of all proteins expressed in human cells) are predicted to be regulated by the miRNA that regulate *CFTR,* and one miRNA can target multiple mRNA, highlighting the complexity of this regulatory system [41]. Thus, other miRNA are likely to act on numerous targets in the NF-κB signalling pathway [42,43], such as miR-9 [44], which has been shown to directly target the 3′-UTR of *RELA* encoding the NF-κB p65 subunit [45] and whose expression increases when NF-κB/MyD88 is activated [46].

Moreover, compensatory phenomena targeting other proteins in this pathway could modulate the regulation of inflammation. Downstream of the NF-κB pathway, miR-93 is controlled by the minichromosome maintenance complex component 7 (*MCM7*) gene, can bind the 3′-UTR of *IL8,* thereby decreasing *IL8* mRNA levels and IL-8 secretion [47]. During *Pseudomonas aeruginosa* infection, miR-93 downregulation has been shown to directly lead to the stabilization of *IL8* mRNA. In CF, NF-κB has been indicated to not be the only transcription factor involved in the regulation of IL-8 secretion; in fact, one study has shown that the MAPK pathway is also implicated in the deregulation of this process, and this activation could compensate the observed decrease in NF-κB activation [33,48,49].

In the future, the role of miR-199a-3p in processes such as apoptosis, proliferation, and differentiation should be determined in the context of CF, as well as its involvement in muscular dystrophy and oesophageal cancer [19,50]. This will allow for the further elucidation of its function in the regulation of cellular processes that involve the MAPK and Wnt pathways. Moreover, the therapeutic use of miR-199a-3p might prevent the chemo-attraction of neutrophils and thus limit the tissue degradation induced by these cells at the pulmonary level. We have demonstrated that miR-199a-3p as a plasma biomarker in the monitoring of pulmonary inflammation in CF patients is not sufficient to improve patient management of inflammatory processes; therefore, more studies are needed to propose specific serum miRNA expression profiles in CF. Nevertheless, this study represents a new breakthrough in our understanding of the regulation of chronic inflammation occurring in CF and highlights the importance of dysregulated miRNA in this regulation.

Data Availability Statement

RNAseq raw datafiles are available in the European Nucleotide Archive (ENA) (primary accession number PRJEB25563). http://www.ebi.ac.uk/ena/data/view/PRJEB25563.

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Author contribution statement

PB performed and designed the experiments, interpreted results, and wrote the manuscript. MDE and FS performed and designed some experiments and interpreted some results. NR and PLR performed some experiments. SBL provided human explants from patients. HC interpreted some results. OT performed and designed experiments, interpreted results, obtained funding, and wrote the manuscript.

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Figure Legends

Figure 1. Flowchart of bioinformatics analyses. Differentially expressed genes/miRNA were initially integrated through correlations of expression data or prediction by target databases. The two approaches were subsequently integrated to identify the most significant miRNA targets. FC = fold-change of absolute values; *p* = *p*-value, Sum = number of databases predicting miRNA and/or mRNA interaction.

Figure 2. Expression of (A) miR-199a-3p, (B) *IKBKB*, and (C) *IL8* in CF (n = 8) and non-CF (n = 9) bronchial tissues. The results are standardized by the expression of endogenous controls *RNU6B* for miRNA and *GAPDH* for *IKBKB* and *IL8*. The Mann–Whitney *U*-test was used to determine significance ($p = 0.0003$, $p = 0.001$, and $p = 0.0003$, respectively).

Figure 3. miR-199a-3p directly regulates IKKβ. (A) Expression of miR-199a-3p measured by RT-qPCR ($p = 0.0002$) in CFBE cells 48 h after transfection with miR-199a-3p mimic or scramble mimic as the control (30 nM; n = 8). (B) Modulation of IKKβ luciferase activity by miR-199a-3p in CFBE cells 48 h after co-transfection of the WT-IKBKB-3'UTR plasmid or M-IKKβ-3'UTR plasmid and miR-199a-3p mimic or scramble mimic as the control (30 nM; n = 4; p=0.0089 and 0.0251). Student's *t*-test was used to determine significance.

Figure 4. miR-199a-3p regulates the NF-κB pathway through IKKβ. Representative western blot of protein expression and quantification of (A) IKKβ (86 kDa; n = 8; $p = 0.0166$), (B) phospho IκBα (39 kDa; n = 5; "n" not significant), and (C) NF-κB (65 kDa) normalized to β-actin (42 kDa; n = 8; $p = 0.0006$), (D) NF-κB activity (n = 5;

p = 0.011), and (E) assay of IL-8 secretion (n = 6; *p* = 0.017) in CFBE cells 48 h after transfection of miR-199a-3p mimic or scramble mimic as the control (30 nM). Student's *t*-test was used to determine significance.

Figure 5. Expression of miR-199a-3p in CF patient and non-CF control plasma. Expression of miR-199a-3p in the plasma of CF patients (n = 25) and non-CF controls (n = 26). The results are standardized by the endogenous plasma control miR-103. Student's *t*-test was used to determine levels of significance.

Table 1. MiRNome and transcriptome results, including significantly differentially expressed (A) miRNA and (B) mRNA in primary air–liquid interface-cultured cells from CF patients and non-CF controls.

A

	microRNA	Log FC	Raw p	padj
Downregulated	hsa-miR-199a-3p	-2.565	5.23E-05	0.026
	hsa-miR-199b-3p	-2.555	5.54E-05	0.026
Upregulated	hsa-miR-449a	1.586	1.87E-04	0.043
	hsa-miR-449b-5p	1.628	1.51E-04	0.040
	hsa-miR-4516	1.733	3.49E-05	0.026
	hsa-miR-449b	1.734	2.19E-04	0.043
	hsa-miR-449c-5p	1.771	1.21E-04	0.036
	hsa-miR-2682-3p	2.124	2.36E-04	0.043
	hsa-miR-449c-3p	2.345	1.82E-05	0.022
	hsa-miR-636	2.533	7.99E-05	0.027
	hsa-miR-639	2.643	2.52E-06	0.006

B

	mRNA	Log FC	Raw *p*	*P*adj	Genes related to the NF-κB pathway
	STC1	-1.48	1.80E-09	2.60E-05	[51]
	UCHL1	-1.73	2.80E-06	9.00E-03	[52]
	FKBP10	-1.64	5.10E-06	1.40E-02	[53]
	SLC25A25	-0.53	6.50E-06	1.40E-02	-
	PDGFRA	-1.13	9.60E-06	1.70E-02	[54]
	ALDH1L2	-1.49	1.20E-05	1.70E-02	[55]
	GFPT2	-1.4	1.90E-05	2.20E-02	[56]
	AC005281.1	-1.33	1.80E-05	2.20E-02	-
	ZEB2	-1.08	2.90E-05	2.80E-02	[57]
	AP000688.8	-1.57	3.50E-05	3.30E-02	-
	RP11-178C3.2	-1.15	4.30E-05	3.60E-02	-
	ZFX	0.64	2.10E-07	1.20E-03	-
	KDM6A	0.65	7.30E-07	3.50E-03	[58]
	SEMA3E	0.8	1.70E-06	7.30E-03	[59]
	RP11-706O15.1	0.81	4.20E-06	1.20E-02	-
	DDX3X	0.44	8.60E-06	1.60E-02	[60]
	HSD17B6	1.25	2.20E-05	2.30E-02	-
	TRAPPC2	0.54	4.10E-05	3.60E-02	[61]

FC = fold-change of absolute values; Raw *p* = raw *p*-value after miRNome or transcriptome analysis; *P*adj = *p*-value adjusted for multiple testing; n = 5 each group.

Table 2. Pearson correlation matrix for miR-199a-3p, *IKBKB*, and *IL8* expression levels in (A) non-CF controls (n = 9) and (B) CF patients (n = 8).

The values in bold are significantly different from 0 at an alpha of 0.05.

A

p-values/non-CF pathology group

Variables	*IL8*	*IKBKB*
miR-199a-3p	**0.001**	**0.002**
IKBKB	**<0.0001**	

B

p-values/CF pathology group

Variables	*IL8*	*IKBKB*
miR-199a-3p	0.115	**0.005**
IKBKB	0.151	

SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Luciferase plasmid construction with the seed sequence of the *IKBK* 3′-UTR. SV40 and CMV: promoters; pUC Ori: origin of bacterial replication for the cloning of the plasmid; ampicillin and neomycin: plasmid selection antibiotics; Gluc-miR target: luciferase reporter gene coupled to the target microRNA target sequence (Gaussia); seAP: alkaline phosphatase gene (endogenous control).

Figure S2. Expression of miR-23a in CF (n = 4) and non-CF (n = 4) bronchial tissues. The results are standardized by the expression of the endogenous control *RNU6B* for miRNA. The Mann–Whitney *U*-test was used to determine significance.

Figure S3. miR-199a-3p transfection concentration determination. Expression of miR-199a-3p after transfection of scramble mimic as the control or miR-199a-3p mimic at concentrations of 2.5, 10, 30, 50, or 100 nM in CFBE cells.

Figure S4. Determination of the time for transfection. Kinetics of chemiluminescent activity of Gaussia and SEAP proteins at 24, 48, and 72 h after transfection of the WT-IKBKB-3'UTR plasmid in CFBE.

Figure S5. Expression of *MTOR* and *ZHX1* in CFBE after transfection with scramble mimic (control) or miR-199a-3p mimic (n = 6; p = 0.0304 and 0.021).