Journal of Cystic Fibrosis 12 (2013) 797 – 802

www.elsevier.com/locate/jcf

Short Communication

Elevated levels of miR-145 correlate with *SMAD3* down-regulation in Cystic Fibrosis patients

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Received 21 November 2012; received in revised form 11 February 2013; accepted 26 March 2013
Available online 28 April 2013

Abstract

MicroRNAs (miRNAs) have recently emerged as important gene regulators in Cystic Fibrosis (CF), a common monogenic disease characterized by severe infection and inflammation, especially in the airway compartments. In the current study, we show that both miR-145 and miR-494 are significantly up-regulated in nasal epithelial tissues from CF patients compared with healthy controls ($p < 0.001$ and $p < 0.01$, respectively) by Quantitative Real-Time PCR. Only miR-494 levels showed a trend of correlation with reduced *CFTR* mRNA expression and positive sweat test values, supporting the negative regulatory role of this miRNA on CFTR synthesis. Using computational prediction algorithms and luciferase reporter assays, SMAD family member 3 (SMAD3), a key element of the TGF-β1 inflammatory pathway, was identified as a target of miR-145. Indeed, miR-145 synthetic mimics suppressed by approximately 40% the expression of a reporter construct containing the *SMAD3* 3′-UTR. Moreover, we observed an inverse correlation between *SMAD3* mRNA expression and miR-145 in CF nasal tissues ($r = -0.68$, $p = 0.0018$, Pearson's correlation). Taken together, these results confirm the pivotal role of miRNAs in the CF physio-pathogenesis and suggest that miRNA deregulation play a role in the airway disease severity by modulating *CFTR* levels as well as the expression of important molecules involved in the inflammatory response. miR-494 and miR-145 may, therefore, be potential biomarker and therapeutic target to specific CF clinical manifestations.

Keywords: Cystic Fibrosis; MicroRNA expression; Post-transcriptional regulation; MiR-145; *SMAD3*; Nasal epithelium

1. Introduction

Cystic Fibrosis (CF) is a common genetic disease caused by mutations in the *CFTR* (Cystic Fibrosis Transmembrane conductance Regulator) gene with more than 1500 pathogenetic variants, the most prevalent being F508del [1]. CFTR is a chloride channel, essential for maintaining sweat, digestive juices and mucus ion balance. *CFTR* mutations affect many organ systems, causing lung inflammation/infection, pancreas insufficiency, intestinal obstruction and male infertility [2]. Thick mucus in the airways is the most common clinical manifestation with recurrent inflammation, chronic microbial infections, followed by injury and deterioration of lung functions [3]. An improper inflammatory profile has been associated with CF, involving deregulation of TGF-β1 signaling pathway via SMAD proteins and aberrant expression of different cytokines, such as interleukin (IL)-8 hypersecretion [4–6]. MicroRNAs (miRNAs) are evolutionarily conserved, small regulatory non-coding RNAs that negatively modulate gene expression at the post-transcriptional level by either repressing translation or decreasing mRNA stability [7]. Dysregulation of miRNA pathways and/or epigenetics-miRNA regulatory circuits has been associated to both multifactorial and

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1569-1993/$ -see front matter
http://dx.doi.org/10.1016/j.jcf.2013.03.007

mendelian diseases [8]. Recent evidences showed the presence of some miRNA-based regulatory circuitry in *CFTR* signaling and CF clinical manifestations [9–12]. We measured the levels of miR-145 and miR-494 in nasal epithelium of a panel of CF patients and healthy controls in order to better define the miRNA role in the CF molecular pathogenesis. Both miRNAs are known as *CFTR* expression post-transcriptional regulators [10,11]. We found that both miRNAs were significantly over-expressed in CF tissues. Moreover, a inverse correlation was evident between the expression of miR-145 and *SMAD3* in CF patients. A trend toward an association was also observed between miR-494 and *CFTR* levels, although it did not reach statistical significance. Our data support the crucial role of miRNAs in the CF pathogenesis by modulating their target gene expression.

2. Materials and methods

2.1. Sample collection

This study is comprised of 18 unrelated patients (mean age 33.1 ± 8.6 years, 11:7 male:female subjects) who received a diagnosis of Cystic Fibrosis at the Regional Center of Cystic Fibrosis—Policlinico Umberto I of Rome, Italy. Patients were eligible if they were 18 years of age or older. CF cases were F508del/F508del homozygotes (11/18) or carried at least one F508del variant: F508del/W1282X (3/18), F508del/N1303K (1/18), F508del/G85E (1/18), F508del/S549R(A > C) (1/18); one individual was homozygote for *CFTR* mutations different from F508del (R553X/N1303K). The segregation of *CFTR* mutated alleles was verified in parents. All CF subjects shared severe lung function impairment with a FEV_1 (forced expiratory volume in 1 s) mean value of 49.2% ± 16.9 and recurrent or chronic pathogen infections, especially *Pseudomonas aeruginosa*. An average value of sweat test, a measure of CFTR-associated functionality, resulted 95.8 ± 15.5 mmol/L. Five non-CF unrelated individuals, without known airway diseases, were used as healthy control group. Italian patients and controls have been fully informed about the aims of our study; all participants freely agreed to take part to the research and signed an institutional written informed consent. The study underwent ethical review and approval according to local institutional guidelines.

2.2. RNA extraction and quantitative Real Time PCR (qRT-PCR)

Respiratory epithelial cells were obtained by brushing each nasal cavity from patients and controls. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse Transcription (RT) for human miR-145 and miR-494 were carried out with TaqMan MicroRNA Assay kit (Applied Biosystem) using 20 ng RNA sample; High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription of total RNA for *CFTR* and *SMAD3* expression analysis. Quantitative Real Time PCR (qRT-PCR) analysis of miRNA and mRNA levels were performed in an Applied Biosystems 7000 Real Time PCR machine using miRNA-specific TaqMan MGB probe (has-miR-145, 002149; has-miR-494, 002365) and TaqMan Gene Expression Assays (*CFTR*, Hs00357011_m1; *SMAD3*, Hs00969210_m1). U6 small nuclear RNA (RNU6) and *GAPDH* mRNA were used as endogenous controls to normalize sample data. PCR reactions were run at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was run in triplicate and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative miRNA or mRNA abundance in the different samples compared to the mean of all control samples represented as unitary value. qRT-PCR data were analyzed using Data Assist software (Applied Biosystems). Experiments were independently carried out three times.

2.3. Bioinformatic analysis

MiR-145 target genes were computationally evaluated using common prediction algorithms provided at TargetScan (release 6.2), miRanda and DIANA-microT-CDS software searching for conserved sites that matched the seed region of the microRNA, obtained from miRBase database. NCBI and UTRdb genome browsers provided information of human *SMAD3* gene (NM_005902; 3HSAR033639).

2.4. Plasmids and luciferase assay

The 3′-UTR region of *SMAD3* (1081-1700 bp and 3100-4223 bp of NM_005902) containing the predicted target sites of miR-145 was amplified from human genomic DNA using a proof reading Phusion High-Fidelity PCR master mix (Finnzymes) with the following primers:

SMAD3 3′UTR-F1 5′-GCTCTAGATCTCCTGAGG TGA AGCTTTTCC-3′ and *SMAD3* 3′UTR-R1 5′-GCTCTA GACCCTGCAGATGAGGCATCAG-3′ for fragment containing "seed 1" and *SMAD3* 3′UTR-F2 5′-GCTCTAGA ATGAGGGCAAGGCTGCTGGC-3′ and *SMAD3* 3′ UTR-R2 5′-GCTCTAGA GTCTGTTTTTACACGCGGC CAC-3′ for "seed 2". The amplified products were inserted into the XbaI site (underlined primer sequences) of the pRLTK vector (Promega). Transformants were validated by restriction digestions and direct sequencing. Luciferase reporter constructs were termed as pSMAD3-S1-3′UTR (620 bp) and pSMAD3-S2-3′UTR (1123 bp). HEK293 cells (1.5×10^5) in 24-well plates were transfected with 50 ng Renilla luciferase reporter vector (pRLTK, pSMAD3-S1-3′ UTR or pSMAD3-S2-3′UTR), 12.5 ng Firefly luciferase control vector (pGL3-SV40, Promega), 100 nM miRNA mimics (hsa-mir-145 miRIDIAN Mimic MI0000461/MIMAT0000437, has-miR-494 miRIDIAN Mimic MI0003134/MIMAT0002816 or miRIDIAN Mimic Negative Control#1 by Dharmacon) and 1.5 μl Lipofectamine 2000 (Invitrogen) following the recommended conditions. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Renilla/Firefly luciferase activity was calculated for each reaction.