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Short Communication

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

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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.
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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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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.

Transfections and luciferase assays were carried out in triplicate and repeated three times.

2.5. Statistical analysis

Results are expressed as mean ± standard deviation (S.D.) from at least three separate experiments. Differences between groups were statistically determined using Student's t test. Correlation coefficients were calculated by Pearson's correlation test. A p-value of less than 0.05 was considered as statistically significant. All statistical analyses were performed using Excel and SPSS software.

3. Results

In order to assess the abundance of miR-145 and miR-494 in total RNA extracted from nasal epithelia of CF patients and healthy control subjects, we performed quantitative Real Time PCR (qRT-PCR) experiments. Indeed, tall columnar epithelial cells have been demonstrated to be valid surrogates for bronchial epithelial cells in airway functional and inflammation studies [13,14]. The miR-relative expression was increased at-least 1.5-fold in 13/18 (72.2%) CF samples for miR-145 and in 14/18 of them (77.8%) for miR-494. As shown in Fig. 1A, the mean levels of both miRNAs were significantly up-regulated in patients compared with controls by around 5 times for miR-145 ($p < 0.001$) and 2.5 times for miR-494 ($p < 0.01$). Interestingly, *CFTR* gene expression in airway nasal tissues, as judged by mRNA level measurements, was down-regulated in CF compared to controls ($p < 0.001$) (Fig. 1B). Using Pearson's correlation test, we analyzed the relationships between miRNA levels and *CFTR* expression. The abundance of *CFTR* transcript was not significantly associated with any miRNA although a trend of inverse correlation was evident with the expression of miR-494 ($r = -0.42$ Pearson's correlation, p = ns) but not with of miR-145 ($r = -0.19$ Pearson's correlation, p = ns). The expression of miR-494 was weakly related with values of sweat test ($r = -0.50$ Pearson's correlation, $p = 0.03$) supporting the observation that this miRNA may be directly involved in the post-transcriptional regulation of CFTR expression and activity [10,11]. In order to identify the potential target(s) of miR-145 in relation with CF, we searched miR-databases using different prediction algorithms such as TargetScan, PicTar and miRanda. The most interesting hit was *SMAD3* gene, which codes for a protein involved in the immunosuppression and anti-inflammatory response mediated by TGF-β1 [15]. As shown in Fig. 2A, *SMAD3* 3′UTR has two putative conserved binding site for the miR-145 seed region at position 1397–1404 (S1) and 3925–3931 (S2). SMAD3 altered expression in different CF model systems, such as *Cftr*$^{-/-}$ mouse nasal epithelium and human tracheal epithelial cells has been extensively reported [4,5]. We generated two different reporter vectors containing either the putative S1 or the S2 binding sites of the human *SMAD3* 3′UTR, downstream of the Renilla luciferase open-reading-frame. When HEK293 cells were transfected with miR-145 mimics (Fig. 2B), we observed a significant reduction in both pSMAD3-3′UTR luciferase activity, compared to microRNA negative control (miR-Ctr). The reduction in luciferase activity was about 40% when pSMAD3-3′UTR-S1 was co-transfected and 30% with pSMAD3-3′UTR-S2 ($p < 0.01$ and $p < 0.001$, respectively). No significant repression was obtained when miR-494 was co-transfected instead of miR-145 confirming the specificity of the miR-responsive elements in the *SMAD3* 3′UTR (Fig. 2B). Considering that miR-145 is up-regulated in CF nasal epithelia and *SMAD3* may be a target for miR-145, we analyzed the expression of *SMAD3* in CF and

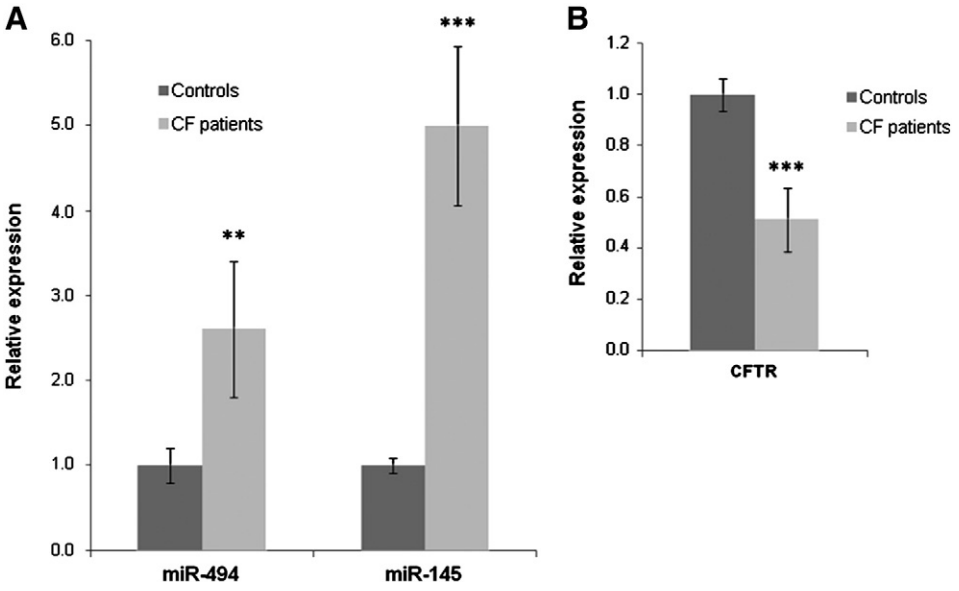

Fig. 1. Up-regulation of miR-145 and miR-494 and down-regulation of *CFTR* mRNA in nasal epithelial cells from CF patients. (A) Comparison of the average expression level of miR-145 and miR-494 between nasal epithelial tissues from CF and non-CF individuals. Data are mean ± S.D. of three separate experiments (**, $p < 0.01$; ***, $p < 0.001$). (B) Relative expression levels of *CFTR* mRNA in nasal epithelial tissues from CF patients compared with non-CF individuals. Data are mean ± S.D. of three separate experiments (***, $p < 0.001$).

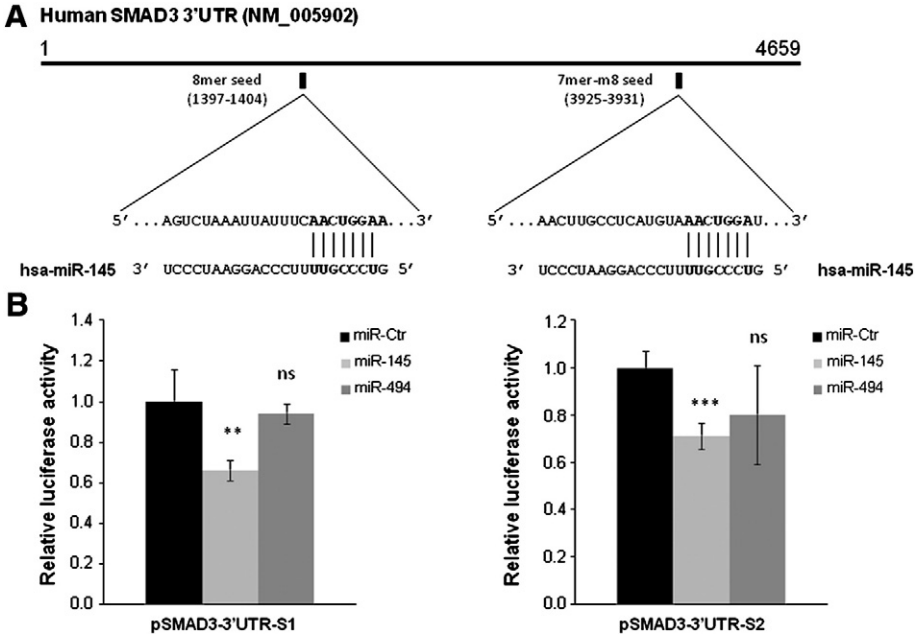

Fig. 2. Mir-145 directly target the *SMAD3* 3′-UTR. (A) Schematic representation of the *SMAD3* 3′UTR and miRNAs (rectangles). Ribonucleotide sequences of the putative miR-145 responsive elements in *SMAD3* 3′UTR paired with the mature human miR-sequence (from TargetScan 6.2 database). Numbers indicate the predicted miR-145 sequences (in bold) using the numbering of the human *SMAD3* 3′UTR (3HSAR033639 from UTRdb database). (B) At 48 h post-transfection, luciferase activity was measured and normalized to the Firefly control in HEK293 cells transfected with pSMAD3-S1-3′UTR or pSMAD3-S2-3′UTR vector together with miR-145 or a negative control miRNA (miR-494 or miR-Ctr). Data are presented as the normalized activity of miR-transfected cells relative to miR-Ctr. These results represent the mean of three independent experiments ± S.D. The significance levels were obtained by Student's t test: **, $p < 0.01$; ***, $p < 0.001$ compared with the control miRNA.

non-CF nasal epithelial tissues. As shown in Fig. 3A, *SMAD3* mRNA levels were significantly down-regulated in airway tissues from CF patients compared with controls ($p < 0.01$). Furthermore, we observed a significant inverse correlation between miR-145 and *SMAD3* mRNA levels ($r = -0.68$ Pearson's correlation, $p = 0.0018$) (Fig. 3B).

In summary, these results show that miR-145 and miR-494 are differently expressed in CF versus non-CF nasal epithelial tissues and, although not statistically significant, a trend for inverse miR-494/*CFTR* relationship is observed. Notably, miR-145 is able to regulate *SMAD3* expression suggesting that an altered SMAD3-mediated inflammatory pathway may be directly correlated to improper levels of miR-145 in CF disease.

4. Discussion

MicroRNAs have emerged as important regulatory molecules involved in a broad range of molecular mechanisms and their deregulation has been implicated in different genetic diseases, including Cystic Fibrosis (CF) [9–12]. We found that miR-145 and miR-494 levels were significantly over-expressed

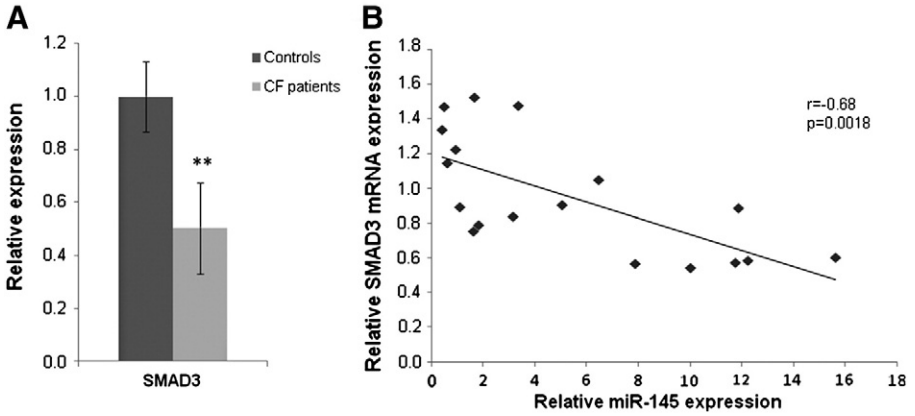

Fig. 3. Down-regulation of *SMAD3* mRNA correlates with elevated levels of miR-145 in CF patients. (A) Reduced of *SMAD3* expression in nasal epithelial tissues from CF and non-CF individuals Histograms represent the average value ± S.D. of three independent experiments (**, $p < 0.01$). (B) Negative correlation between miR-145 and *SMAD3* mRNA expression levels in samples from 18 CF patients (Pearson's correlation analysis).

in nasal epithelia from CF patients compared to healthy individuals. Specifically, miR-494 levels were about 2.5-fold higher in CF samples with a trend for inverse correlation between this miRNA and *CFTR* levels. Although not statistically significant, this evidence suggests that miR-494 may behave as a genetic modifier for the CF clinical manifestations also modulating the *CFTR* gene expression, as an additional level of control over *CFTR* mutations. These results are in agreement with the observations that miR-494 mimic overexpression is able to directly suppress *CFTR* mRNA [10,11]. Beside the common F508del variant, our CF patients carry missense and nonsense alterations in the *CFTR* gene. Indeed, different studies have shown that the effect of nonsense mutations on *CFTR* mRNA levels are very variable [16] and nonsense-mediated decay (NMD) is addressed as the major mechanism for rapid degradation of aberrant transcripts harboring premature termination codons. Interestingly, microRNAs have recently been defined as important regulators of the NMD efficiency [17]. So, aberrant expression of specific miRNAs in CF patients could directly or indirectly have important implications on *CFTR* mRNA levels. Up-regulation of miR-494 in asthmatic samples [18] and direct influence in cell cycle progression of primary murine bronchial epithelial cells exposed to carcinogens [19] suggest that miR-494 altered levels have effects mainly in the lung compartment.

We also observed that a second miRNA, miR-145, was increased in nasal airway cells of CF versus non-CF individuals. The expression increase of miR-145 was significantly correlated with a consistent decrease of *SMAD3* mRNA, a potential target of this miRNA. SMAD3 is a key component of the immune system which plays a protective effect against infection and inflammation [15]. *SMAD3* expression is decreased in CF nasal epithelial cells [5,6] suggesting a correlation with the associated increase of miR-145. The direct silencing of *SMAD3* gene provides a possible explanation for the improper inflammatory response and to such an airway remodeling/fibrosis as common in CF patients. Since SMAD3 has been proposed to play a role in the TGF-β1-mediated negative regulation of the NF-kB/IL-8 pathway [20], a diminished SMAD3 expression may contribute to the hyperinflammation observed in the CF airways. The relationship between miR-145 and *SMAD3* is well demonstrated by the luciferase assays showing a meaningful decreased activity of *SMAD3* 3′UTR-reporter constructs after miR-145 transfection. The reduced expression of *SMAD3* is consistent with previous findings in cultured cell models of CF as well as in the nasal epithelium of *cftr*$^{-/-}$ mice [4] and suggests that miR-145 control pathway could represent a potential therapeutic target aimed to restore SMAD3-mediated TGF-β1 signaling abnormalities identified in CF.

In conclusion, the present study demonstrates the importance of miRNA-related pathways may have as modulatory factors in CF clinical manifestations. In particular, the discovery of miR-145 and miR-494 altered levels in CF patients and the evidence of a strong inverse correlation with *SMAD3* gene expression suggests the possibility of a coordinated network of gene expression control by microRNAs. miR-145 and miR-494 expression seem to have particular importance in airway tissue functions, as potential modulators pathological events, such as increased susceptibility to infections, chronic airways inflammation and response to specific therapies. Future studies will be planned to determine the molecular pathways of miR-494/miR-145 in cell line models and to analyze the correlation between miRNA abundance and specific symptoms in a wide cohort of CF patients, especially in CF relatives (twins or siblings) sharing the same *CFTR* pathological alleles but different phenotypes. Ameliorating the acknowledgments of miRNA regulators, downstream targets and signaling pathways in Cystic Fibrosis is essential for elucidating complex regulatory networks that are critical for the disease biology and severity. The identification of CF-related microRNAs potentially offer novel therapeutic applications to ameliorate specific clinical aspects and response to therapy in disease complications.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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