miR-125b/NRF2/HO-1 axis is involved in protection against oxidative stress of cystic fibrosis: A pilot study

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Abstract. In the physiopathology of cystic fibrosis (CF), oxidative stress implications are recognized and widely accepted. The cystic fibrosis transmembrane conductance regulator (CFTR) defects disrupt the intracellular redox balance causing CF pathological hallmarks. Therefore, oxidative stress together with aberrant expression levels of detoxification genes and microRNAs (miRNAs/miRs) may be associated with clinical outcome. Using total RNA extracted from epithelial nasal cells, the present study analyzed the expression levels of oxidative stress genes and one miRNA using quantitative PCR in a representative number of patients with CF compared with in healthy individuals. The present pilot study revealed the existence of an association among CFTR, genes involved in the oxidative stress response and miR-125b. The observed downregulation of CFTR gene expression was accompanied by increased expression levels of Nuclear factor erythroid derived-2 like2 and its targets NAD(P)H:Quinone Oxidoreductase and glutathione S-transferase 1. Moreover, the expression levels of heme oxygenase-1 (HO-1) and miR-125b were positively correlated with a forced expiratory volume in 1 sec (FEV1) >60% in patients with CF with chronic *Pseudomonas aeruginosa* lung infection (r=0.74; P<0.001 and r=0.57; P<0.001, respectively). The present study revealed the activation of an inducible, but not fully functional, oxidative stress response to protect airway cells against reactive oxygen species-dependent injury in CF disease. Additionally, the correlations of HO-1 and miR-125b expression with an improved FEV1 value suggested that these factors may synergistically protect the airway cells from oxidative stress damage, inflammation and apoptosis. Furthermore, HO-1 and miR-125b may be used as prognostic markers explaining the wide CF phenotypic variability as an additional control level over the CFTR gene mutations.

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Introduction

Large variations in CF phenotypes observed among the patients cannot be simply explained by the CFTR mutations since, frequently, patients with identical genotype show varying severity of clinical presentations even within the same family. Indeed, modifier genes and environmental factors are likely to modulate the disease severity.

Over the past few years, microRNAs (miRNAs) have been suggested to act as modulators of CF disease severity. Aberrant levels and functions of these molecules have been observed in CF tissues (1). miRNAs are small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level by repressing translation or decreasing mRNA stability (2). Mature miRNAs are able to bind target transcripts through base pairing with their 3'-untranslated regions (3'UTRs). Recently, some studies have investigated differentially expressed miRNAs in CF cells: miR-126 that regulates the expression of Target Of Myb1 protein 1 (3), which in turn is a negative regulator of the Interleukin 1 beta (IL1β) and tumor necrosis factor alpha (TNFα) signal pathways (3); miR-155, implicated in the phosphoInositide 3-kinase/protein kinase B pathway, which leads to the expression of the proinflammatory cytokine IL8, thus contributing to exaggerated inflammation observed in CF patients (4); miR-145, which is able to regulate the expression of Smad family member 3 (SMAD 3), a component of the Transforming Growth Factor beta (TGFβ) inflammatory pathway (5). Altogether, these results support the notion that changes in miRNAs may contribute to CF clinical phenotype, also considering the important regulatory role of these molecules in the expression of a wide range of genes (1,6), whose deregulation has been implicated in different disease (7-9).

Oxidative stress is a biological response with key role in a variety of inflammatory diseases. It is caused by an imbalance between antioxidants and radical oxygen species. When oxidative stress occurs, cells try to counteract its effects and to restore the redox balance by modulating genes encoding defensive enzymes,

transcription factors, and structural proteins. The master gene of this cellular defense response is nuclear factor erythroid derived-2 like2 (NRF2), a transcription factor that positively regulates genes encoding proteins implicated in detoxification. Inactive NRF2 is restrained in the cytoplasm associated with Kelch-like Ech-associated protein 1 (KEAP1). Upon exposure of cells to oxidants, it is phosphorylated and translocated to the nucleus where it binds the antioxidant response elements within the promoter of target genes and transactivates them.

Oxidative stress resulting in ROS increase is often observed in several human diseases. CF is characterized by the disruption of many processes as aberrant ion transport, inflammation responses, metabolism of lipids and proteins that are modulated by oxidants and antioxidants. In CF context, defective function of CFTR prompts to produce a redox imbalance in epithelial cells and extracellular fluids and causes an abnormal flux of ROS. The occurrence of ROS increase is dependent on a diminished availability of important dietary antioxidants, such as vitamin E and carotenoids, as a result of maldigestion, malabsorption, increased turnover (10) and sustained activation of neutrophils (11). In this disease, a suboptimal antioxidant protection is a main contributor to oxidative stress together with a poor control of immuno-inflammatory pathway.

As previously reported, cellular response to ROS is carried out by transcriptional activity of NRF2 which induces some deoxidant ARE (antioxidant responsive elements) genes, which in turn help to recover cellular homeostasis. In different cell context, the well-known and studied targets are: NAD(P)H:Quinone Oxidoreductase (NQO-1), glutathione S-transferase 1 (GST-T1) and heme oxygenase (HO-1).

Specifically, HO-1 is strongly upregulated during stress and exerts its function by catabolizing heme to biliverdin, free iron and carbon monoxide to counter sequential imbalance. Its expression was demonstrated cytoprotective against lung injury of oxidative stress and inflammation not only in CF patients (12) but also in subjects with other lung diseases (11). HO-1 prompts to a better prognosis by protecting the airway epithelial and endothelial cells and pancreatic beta cells from injury and apoptosis (13,14).

Recently, some studies have shown that NRF2 directly regulates the expression of different miRNAs at transcriptional level, including miR-125b (15,16). miR-125b is a highly conserved homolog of *lin-4* and, recently, it has been found to be modulated by CFTR-mediated HCO_3^- influx through the sAC-PKA-NFKB cascade in mice embryo development (17), this suggesting a role of epigenetic regulator of CFTR in addiction to the anion channel. Moreover, in a genetic condition affecting keratinocytes, our previous studies showed that miR-125b expression is regulated by oxidative stress-dependent mechanisms (18). As miRNAs are thought to function both as drivers and modifiers of CF phenotype and several studies highlighted that these small molecules can regulate and be regulated by oxidative stress (19), we hypothesized that miR-125b is a good candidate to play an important role in the CF pathophysiology.

All these remarks prompted us to investigate whether oxidative stress may represent a mechanism involved in the CF pathogenesis, given its role in the CF lung disease. In this pilot study we analyzed a possible correlation among miR-125b, NRF2 together with its targets and CFTR expressions in 13 CF patients. Indeed, the validation of the miR-125b/NRF2/CFTR circuitry role in CF disease may lead to the identification of new therapeutic strategies against oxidative stress along with airway inflammation in CF patients.

Materials and methods

Sample collection. We analyzed 13 unrelated CF individuals (mean age, 30.77 years, 8:5 male:female patients) who received a diagnosis of Cystic Fibrosis at the Regional center of Cystic Fibrosis-Policlinico Umberto I of Rome, Italy. Patients were F508del/F508del homozygotes and were eligible if they were 18 years old or older. The segregation of CFTR mutated alleles was verified in parents. CF subjects are stratified into two groups: Group 1 (P1-P4) shared mild lung function impairment with a FEV1 mean value 79.5% without *P. aeruginosa* (PA) chronic infection (PA[-]); group 2 (P5-P13) shared recurrent or chronic pathogen infections (PA[+]) with FEV1 mean value 53.78%. Furthermore, group 2 PA[+] were stratify in two subgroups based on the best or worse FEV1: FEV1>60% or FEV1<60%, respectively. The values of sweat test, a measure of CFTR-associated functionality, resulted higher than 30 mmol/l. Three non-CF unrelated individuals, without known airway diseases, were used as healthy control group.

RNA extraction and reverse transcription (RT). By cytobrushing, primary nasal epithelial cells were isolated from nasal cavity of patients and controls. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RT for miR-125b was carried out with TaqMan MicroRNA Assay kit (Thermo Fisher Scientific) using 20 ng of RNA sample; High capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) was used for RT of total RNA for CFTR and NRF2 expression analysis using 500 μg-1 mg of RNA sample (20).

Quantitative Real Time (qPCR). CFTR, NRF2, HO-1 and NQO1 mRNAs and miR-125b levels were analyzed by StepOnePlus Real-Time PCR System machine using Taqman Gene expression Assay (CFTR Hs00357011; NRF2 Hs 00975961_g1; HO1, Hs01110250_m1; NQO1, Hs02512143_s1; miR-125b, hsa-miR125b 000449) with TaqMan Universal PCR Master Mix II (Thermo Fisher Scientific). GAPDH (Hs02758991_g1) mRNA and U6 small nuclear RNA (Hs001973) were used as endogenous controls to normalize sample data. SYBR green fluorescence chemistry for GST-T1 and GAPDH expression analysis were carried out using specific primers (GST-T1 Fw: AAGGTCCCTGACTACTGG TA; GST-T1 Rev: ATACTGGCTCACCCAGGAAA; GAPDH Fw: TGCACCACCAACTGCTTAG; GAPDH Rev: GAG GCAGGGATGATGTTC) with SensiFAST SyBr Hi-ROX kit (Bioline, UK). The fold change of the mRNA gene and miRNA in the CF samples relative to the mean values of healthy control samples was calculated using $\Delta\Delta$Ct method (21,22).

Statistical analysis. Results of real time PCR are expressed as mean standard deviation (SD) from at least three separate experiments and P-value was used for significance using Student's t-test. Correlation coefficients were calculated by Pearson's correlation test. All statistical analyses were performed using Excel software.

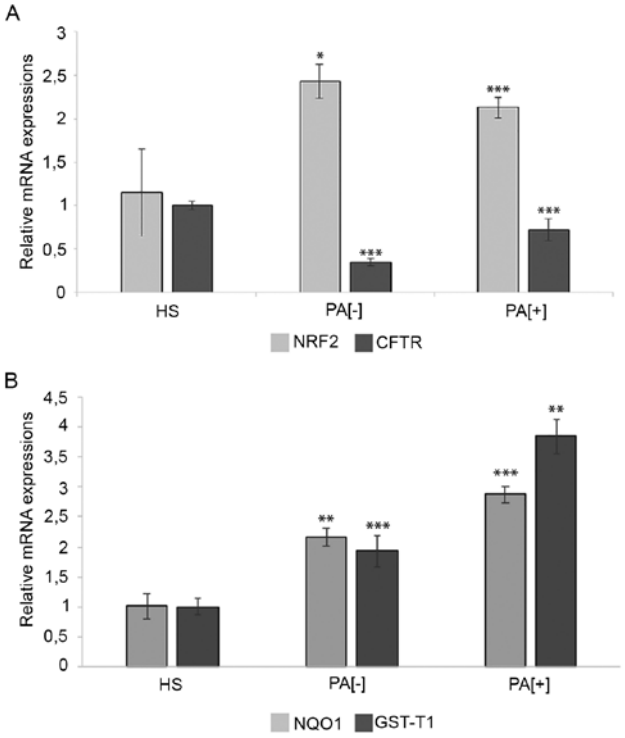

Figure 1. Relative mRNA expression levels of CFTR and oxidative stress genes. (A) Upregulation of NRF2 together with downregulation of CFTR in patients with CF with or without PA chronic infection (PA[+] and PA[-], respectively) compared with in HS. (B) Upregulation of the expression levels of NQO1 and GST-T1 in PA[-] or PA[+] patients with CF compared with in HS. The data are presented as the mean ± SD of three independent experiments. *P<0.05; **P<0.01; ***P<0.001 vs. respective HS group. PA, *Pseudomonas aeruginosa*; CF, cystic fibrosis; HS, healthy subjects; CFTR, cystic fibrosis transmembrane conductance regulator; NRF2, nuclear factor erythroid derived-2 like2; NQO1, NAD(P)H:Quinone Oxidoreductase; GST-T1, Glutathione S-Transferase 1.

Results

We analyzed CFTR and NRF2 mRNA levels together with its target expressions in CF patients compared to non-CF healthy individuals (n.3), by qPCR using TaqMan chemistry. The lost function of CFTR generally correlates with increased oxidative stress caused by impaired antioxidant response (23). Interestingly, qPCR analysis showed doubled mRNA levels of the master detoxification gene NRF2 (Fig. 1A) in both patient groups PA[-] and PA[+] (P-value=0.018 and 0.00013, respectively) and so might be used as marker of oxidative stress onset. Moreover, in line with these data, the expression levels of NRF2 targets, NQO1 (P-value=0.0079 and 0.00084 respectively) and GST-T1 (P-value=0.00029 and 0.0016, respectively) were also upregulated (Fig. 1B). These results support the hypothesis that oxidative stress induces the transcriptional activity of NRF2, which in turn activates NQO1 and GST-T1 though this protective cascade failed to rescue anyhow the unbalanced redox microenvironment, possibly because ROS levels are too high to be counteracted in CF patients.

Furthermore, we evaluated expression profiles of HO-1 and miR-125b. Interestingly, we observed not significantly changed expression neither in HO-1 deoxidant enzyme nor in miR-125b in PA[-] patients (data not shown). Instead, after further stratification of PA[+] patients in mild or severe phenotype group, based on FEV1>60% and FEV1<60% respectively, the analysis displayed induced expressions of HO-1 and miR-125b mainly in the first group than in the second (P-value=0.015 and 0.0023, respectively) (Fig. 2A and B).

To further confirm these results, using the Pearson's correlation test, we showed that the abundance of HO-1 and miR-125b transcripts was directly and significantly associated with FEV1: r=0.74 P<0.001 and r=0.57 P<0.001, respectively (Fig. 2C and D).

We speculated that the concomitant upregulation of HO-1 and miR-125b might supply a synergistic protective effect, which might promote a better prognosis in CF patients by attenuating ROS-dependent damage and reducing apoptotic signals.

Table I. Clinical characteristics of patients with cystic fibrosis (n=13).

Characteristic	Value
Sex, male:female ratio	8:5
Mean age ± SD, years	30.77±6.70
Genotype	F508del CFTR
Positive for *Pseudomonas aeruginosa* chronic colonization, n	9
Negative for *Pseudomonas aeruginosa* chronic colonization, n	4

CFTR, cystic fibrosis transmembrane conductance regulator.

Discussion

Oxidative stress in Cystic Fibrosis has been widely demonstrated. Because of a defective CFTR channel, redox imbalance develops and in turn induces the increase of ROS with all well-known effects.

In particular, CF lung displays a chronic inflammation that sustains the recruitment of neutrophils to destroy pathogenic bacteria through phagocytosis and release of cytokines, resulting in the release of large amount of ROS and other immunological mediators. This increased oxidant load produces a redox imbalance which affects antioxidant capacity of the cells, thus resulting in oxidative insults. Moreover, *Pseudomonas aeruginosa* infection produces pyocyanin (N-methyl-1-hydroxiphenazine), a redox-active virulence factor that further increases ROS occurrence (24). Lastly, high ROS amount causes cell death through apoptosis or necrosis.

For basic research studies the *in vitro* cell models play a key role. The best physiological model of airway epithelium are primary bronchial epithelia, but this kind of cells are limited due to human lung tissue availability. Fortunately, it has been shown primary nasal cells represent a valid alternative model for CF pathophysiology studies summarizing the properties of bronchial cultures and have been used in different airway functional and inflammation studies (25-27).

We extracted mRNA from nasal epithelial cells of 13 ΔF508 CF patients stratified in low risk (P1-P4 PA[-]) or high risk (P5-P13 PA[+]) (Table 1).

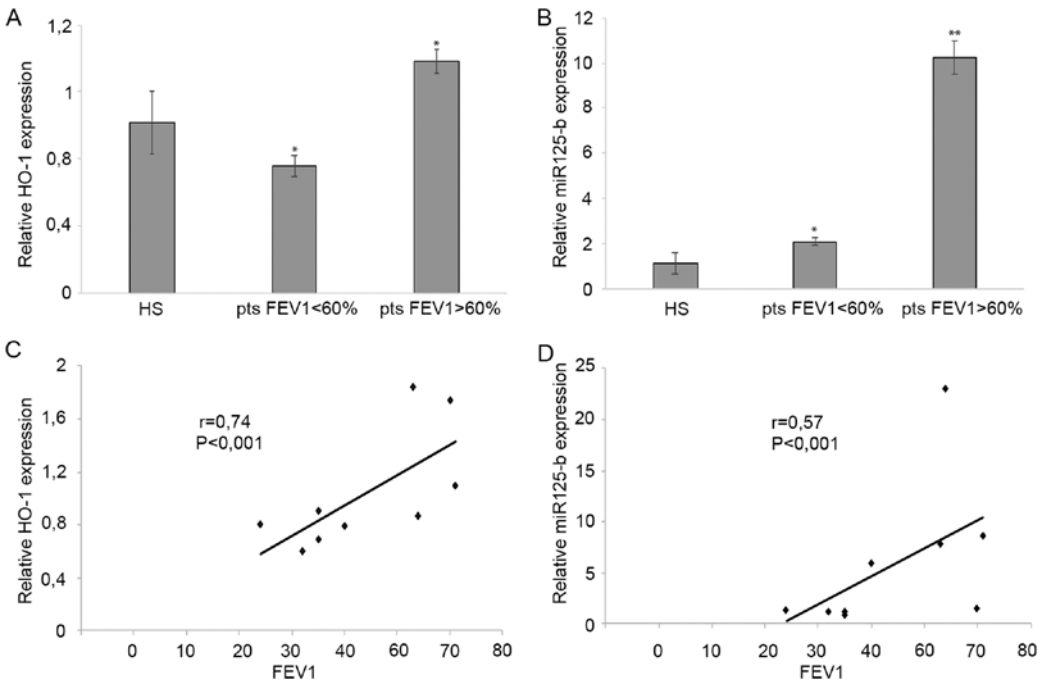

Figure 2. Upregulation of HO-1 and miR-125b expression is correlated with an improved FEV1 in PA[+] patients with CF. Relative expression levels of (A) HO-1 and (B) miR-125b in PA[-] or PA[+] patients with CF compared with in HS. The data are presented as the mean ± SD of three independent experiments. Positive correlation between (C) HO-1 and (D) miR-125b mRNA expression with FEV1 in samples from 9 PA[+] patients with CF, analyzed via Pearson's correlation analysis. *P<0.05 and **P<0.01 vs. HS. PA, *Pseudomonas aeruginosa*; CF, cystic fibrosis; HS, healthy subjects; FEV1, forced expiratory volume in 1 sec; miR, microRNA; HO-1, heme oxygenase-1; pts, patients.

We found that NRF2 mRNA expression was doubled, together with CFTR downmodulation, in nasal epithelia from ΔF508 CF patients compared to healthy subjects. NQO-1 and GST-T1, two direct targets of NRF2, resulted overexpressed also.

NRF2 mRNA is expressed broadly and independently of inducers sustaining a post-transcriptional mechanism for its activation, so it might be important show the correlation between the protein and the RNA expressions of CFTR and NRF2. Unfortunately, we are not able to perform protein extraction or FACS analysis because of too low amount of the obtained nasal cells with cytobrush.

However, our results imply the onset of oxidative stress that cells are unable to neutralize due to the too high levels of ROS. Moreover, our results suggest that a protective circuitry might be functional, but not sufficient to maintain physiological levels of ROS, which would allow to avoid oxidative injury in CF context.

Then we analyzed the expression values of two additional NRF2 targets: HO-1 and miR-125b. HO-1 gene encodes an antioxidant enzyme that is inducible in response to ROS, proinflammatory cytokines or endotoxins (13). Its action is carried out through degradation of heme into bilirubin, Fe^{2+} and CO, so modulating the oxidative damage. Moreover, Heme-oxygenase was demonstrated to be involved in cytotoxic protection against cellular lesion induced by *P. Aeruginosa in vitro* in CF patients (12) as well as active against oxidative injury not only in lung-affected diseases [CF, chronic Obstructive Pulmonary Disease (COPD), idiopathic pulmonary fibrosis (IPF)] but also in other diseases, as neurodegenerative or cardiovascular, and in hematological malignancies (28-30). Furthermore, invasive *P. aeruginosa* strains have been demonstrated to induce mitochondrial apoptosis in target host cells through some virulence factors (31) and HO-1 resulted defensive against apoptosis because oxidative insults prompt a broad range of effects like growth arrest, senescence and cell death.

The present results displayed that the high expression of HO-1 was positively correlated with FEV1>60% in airway epithelial cells of PA[+] patients, this indicating that the stress response player might protect cells from oxidative stress injury. Accordingly, in lung aberrant inflammation HO-1 may act as a modifier gene able to affect CF clinical manifestations.

Recently, multiple studies are exploring the mechanisms of miRNAs as pivotal modulators of cellular activities mostly in association with many diseases. In particular, some studies have shown that miR-125b was regulated by NRF2 through binding on ARE within its promoter region (15,16); then, specifically, other studies showed that CFTR is able to modulate miR-125b expression through HCO_3 influx (17). In addition, our previous results demonstrated the involvement of oxidative stress in miR-125b modulation in skin disease (18) and not least, in apoptotic signaling repression targeting p53 in rat models (32).

Furthermore, we have found that miR-125b was significantly overexpressed in PA [+] patients with better lung function. The basic defect caused by CFTR mutations, recurrent bacterial infections, impaired antioxidant protection system and all hallmarks of CF disease bring excessive ROS production. This ROS overload induces DNA, protein and lipids damage, inflammation and apoptosis (23) so we speculated that miR-125b might be protective of airway cells against apoptosis, but future investigations need to demonstrate this miRNA function in CF disease.

In summary, by qPCR analysis on airway epithelial cells of CF patients we have demonstrated that oxidative stress response mechanisms are activated but fail to counteract it; nevertheless, synergistic effects of HO-1 and miR-125b might have protective effect, which is involved in phenotypic variability of CF disease and oxidative stress-related genes might be potential biomarkers for better prognosis. Until now there are still few biomarkers demonstrated to be predictive of CF clinical outcome. Thus, it's very important the discovery of clinically relevant factors able to support the diagnosis, to forecast the progress of disease and to understand the impact of therapies. The identification of valid biomarkers might help clinicals to treat early patients to avoid recurrent lung exacerbations generally involved in decline of medical case.

Future studies will be addressed to further confirm these results in a wide cohort of CF individuals as well as in CF *in vitro* models in order to examine the molecular pathways of the potential cross-talk between of HO-1 and miR-125b in protecting airway cells from oxidative stress and apoptosis, finally sustaining their contribution in CF clinical manifestations.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SC conceived and designed the experiments, and wrote the manuscript. MP and SC performed the experiments. SC, MP and DS analyzed and interpreted the data. CT and IS provided facilities, performed the data analysis and critically revised the manuscript. SQ, GC and AP acquired and managed patients, provided clinical information in interpreting experimental data and critically revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Sapienza University, Policlinico Umberto I (Rome, Italy). Patients with cystic fibrosis and healthy controls were fully informed of the aims of the present study and freely agreed to take part to the research by signing an institutional written informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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