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RNA editing applied to cystic fibrosis: RESTORE can target G542X CFTR mRNA and revert the nonsense mutation

Simona Titoli [a], Viviana Barra [a,*], Serena Gargano [a], Aldo Di Leonardo [a,b], Raffaella Melfi [a]

[a] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo 90128, Italy
[b] Centro di Oncobiologia Sperimentale (C.O.B.S.), Viale Delle Scienze, Palermo 90128, Italy

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ABSTRACT

Background: Nonsense mutations in the CFTR gene are responsible for approximately 8 % of cystic fibrosis (CF) cases worldwide. The consequent premature termination of translation leads to the production of a truncated and non-functional CFTR protein. Despite the intensive research in the field, these patients cannot benefit from specific and approved therapies yet. To address this issue, in this study we evaluated a potential therapeutic strategy to overcome the nonsense G542X (UGG > UGA) mutation in the CFF-16HBEge human bronchial epithelial cells by restoring the full-length CFTR protein.
Methods: We applied the RESTORE (Recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing) approach, based on specifically designed antisense RNA oligonucleotides (ASOs) to recruit endogenous ADAR (adenosine deaminase acting on RNA) enzymes. The ADAR's recruitment to the target CFTR mRNA is expected to promote the deamination of adenosine (A) into inosine (I) within the premature termination codon (UGA). As the ribosome reads the inosine as guanosine (G), the stop codon could be recoded as a tryptophan (UGG), thereby allowing the synthesis of a full-length CFTR protein, albeit with a different amino acid.
Results: Our results indicate that in the CFF-16HBEge G542X cell line, the transfection of a specific ASO allows the rescue of the CFTR transcript and protein expression, compared to the untransfected mutated cells. Next generation sequencing of CFTR cDNA also confirmed the occurrence of the expected RNA editing outcome.
Conclusions: The obtained results suggest that the RESTORE approach might be explored as a promising strategy to treating nonsense mutations in *CFTR*, potentially contributing to novel therapeutic options for CF patients.

1. Introduction

CF is a monogenic autosomal recessive disease caused by mutations in the CF transmembrane-conductance regulator gene (*CFTR*) located on chromosome 7 (7q31.2) (Wang, 2023). More than 2,100 mutations have been identified in the CFTR gene ("Cystic Fibrosis Mutation Database") and, among these, around 8.4 % are nonsense mutations ("Cystic Fibrosis Mutation Database"), classified as Class I mutations (e.g., CFTR-W1282X and −G542X). CFTR nonsense mutations are considered amongst the most severe, since they cause truncation of the CFTR protein and instability of the CFTR mRNA (Wang, 2023). Nonsense mutations introduce a premature stop codon (PTC) in the gene, leading the CFTR mRNA to be degraded through the nonsense-mediated decay (NMD) pathway (de Poel et al., 2022). Any CFTR mRNA that escapes from NMD can be translated by ribosomes into a shortened and non-functional CFTR protein which is then degraded by the proteasome (Pranke et al., 2019). Recent advancements in CF therapy lie in the advent of CFTR modulators, designed to target and modulate the

Abbreviations: CFTR, cystic fibrosis transmembrane-conductance regulator; CF, cystic fibrosis; RESTORE, recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing; ADAR, adenosine deaminase acting on RNA; ASO, antisense oligonucleotide; PTC, premature termination codon; NMD, nonsense-mediated decay; TRIDs, translational readthrough-inducing drugs; ZFN, zinc finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein; REPAIRv2, RNA editing for programmable A to I replacement, version 2; mxABE, mini-dCas13X-mediated RNA adenine base editor; PS, phosphorothioate; 2′-OMe, 2′-*O*-methylated; LNA, locked nucleic acid; IF, immunofluorescence; NGS, next generation sequencing; FDA, food and drug administration; nNOS, nitric oxide synthase.

* Corresponding author.
E-mail addresses: simona.titoli@unipa.it (S. Titoli), viviana.barra@unipa.it (V. Barra), serena.gargano01@unipa.it (S. Gargano), aldo.dileonardo@unipa.it (A. Di Leonardo), raffaella.melfi@unipa.it (R. Melfi).

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dysfunctional CFTR protein (e.g. ivacaftor, lumacaftor, tezacaftor, elexacaftor) (Regard et al., 2023). Unfortunately, these therapies are ineffective for patients with nonsense mutations, as they do not have any CFTR protein (Wang, 2023) to rescue. Therefore, exploring new approaches to target nonsense mutations is necessary to widen therapeutic possibilities. Many studies have revealed that small molecules acting as translational readthrough-inducing drugs (TRIDs), can partially restore CFTR functionality by allowing the ribosome to pass through a PTC in the mRNA and continue translation by inserting a near-cognate amino acid (Lentini et al., 2014; de Poel et al., 2022; Sharma et al., 2021). The potential off-target effects, the mechanisms of action, and the safety of TRIDs are still under investigation (Carollo et al., 2023; Corrao et al., 2022; Perriera et al., 2023). Currently, other genetic therapies that are being explored involve gene editing, for example by zinc finger nuclease (ZFNs), transcription activator-like effector nuclease (TALENs) or by clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas)-based tools (Marina et al., 2020), aiming to repair the mutant CFTR DNA in its natural location on the chromosome, resulting in the production of functional CFTR protein (Harrison, 2022). Other promising methods have focused, instead, on mRNA therapy and editing (Harrison, 2022; Rang et al., 2020). Adenosine to inosine (A-to-I) editing is a common post-transcriptional RNA modification that occurs in a large variety of organisms, including humans (Nishikura, 2016), to precisely modify genetic information, without changing genome sequences (Han et al., 2022). A-to-I editing is catalysed by the adenosine deaminase acting on RNA (ADAR) enzymes (Montiel-Gonzalez et al., 2013) that recognize the target adenosine in perfectly or partially double-stranded RNAs. This mechanism can be exploited to specifically edit mutant RNAs, and since inosine is decoded as guanosine by ribosomes, PTCs (UGA or UAG) can be converted in a coding codon UGG. Indeed, different RNA editing tools have been developed that are based on the use of exogenous or endogenous ADARs (Bellingrath et al., 2023; Montiel-Gonzalez et al., 2013). Two systems exploiting exogenous enzymes were recently described, the REPAIRv2 (RNA editing for programmable A to I replacement, version 2) system (Cox et al., 2017) and the mxABE (mini-dCas13X-mediated RNA adenine base editor) system (Xu et al., 2021). Previously, we demonstrated that the two platforms were successful in editing nonsense CFTR mRNA mutations in cultured CFF-16HBEge (W1282X and G542X) (Chiavetta et al., 2023; Melfi et al., 2020). Alternatively, it is possible to engage the endogenously expressed ADAR enzymes to modify a specific nucleotide (Bellingrath et al., 2023). The second approach would be more suitable for therapeutic applications because it eliminates the need to introduce exogenous proteins, which could trigger immune responses or exhibit unpredictable behavior. For instance, the strategy known as RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide mediated RNA editing), recently described by Merkle and colleagues (Merkle et al., 2019), is based on the use of antisense RNA oligonucleotides (ASOs) as guides to recruit endogenous ADARs to specific sites. These ASOs consist of two parts, a variable sequence of chemically modified nucleotides, complementary to the mutated region of the target RNA sequence, and an invariant domain of 55 nucleotides forming an ADAR-recruiting hairpin. A cytidine-adenosine mismatch allows the recognition of the target adenine by ADARs, which catalyse the deamination of adenosine into inosine. When editing occurs in a PTC, the ribosome reads the inosine as guanosine, allowing the translation process to continue and to restore full-length protein expression (Montiel-Gonzalez et al., 2013). These ASOs are chemically modified to enhance protection against nuclease degradation and to facilitate their passage through the plasma membrane without requiring a delivery vector (Quemener et al., 2020). Merkle and colleagues showed that these chemically modified ASOs preferentially promote RNA editing by the enzyme ADAR1 over ADAR2, specifically, favoring the ADAR1 p150 isoform (Merkle et al., 2019). This isoform can shuttle between the nucleus and the cytoplasm, allowing it to perform RNA editing in both cellular compartments. However, ADAR1 p150 preponderant localization is in the cytoplasm, where, in presence of the ASOs, is crucial to promote the expected RNA editing events (Merkle et al., 2019; Patterson and Samuel, 1995). Here we investigated the possibility of applying the RESTORE system to edit CFTR mRNA and correct PTCs. We show that this approach partially recovered the CFTR protein in the human immortalized bronchial epithelial CFF-16HBEge cells isogenic for the G542X (c.1624G>T) stop mutation (hereafter referred to as $16HBE^{G542X}$) (Valley et al., 2019). These results suggest the potential for the RESTORE strategy in editing of the CFTR mRNA with the G542X nonsense mutation, which deserves to be further explored to offer a glimmer of hope for treating cystic fibrosis patients harboring this mutation (Booth et al., 2023; Han et al., 2022). This, however, also would imply that the ASOs-recruiting ADAR enzymes and the subsequent induction of RNA editing decreases over time, requiring repeated dosing to sustain therapeutic levels and maintain the CFTR protein levels (Gold et al., 2021). Therefore, further studies are needed to investigate the long-term efficacy of ASOs.

2. Materials and methods

2.1. Antisense RNA oligonucleotides design

ASOs were designed *ad hoc* to target endogenous ADARs to the adenine in positions 1,626 in the CFTR mRNA and synthesized by Eurogentec (Seraing, Belgium). The 5′ ASO sequence corresponds to the invariant 55 nucleotides forming the ADAR recruiting domain, followed by the 3′-specific domain that spans 40 nucleotides complementary to the mutated region of the target CFTR mRNA sequence. The cytidine-adenosine mismatch maps at nucleotide 11 from the 3′ end of the ASO. The following modifications were introduced in the sequences, two 5′ and four 3′ terminal phosphorothioate (PS) bonds, 2′-*O*-methylated (2′-*O*Me) ribonucleotides and locked nucleic acid (LNA) nucleotides (Fig. 1A) (Aquino-Jarquin, 2020; Merkle et al., 2019).

2.2. Cell culture conditions

The $16HBE^{G542X}$ cell line, provided by the Cystic Fibrosis Foundation (Lexington, MA, USA) and the parental 16HBE14o- bronchial epithelial cell line (Valley et al., 2019), used as a control for wild-type CFTR, were grown in PureCol (Cell Systems GmbH, Troisdorf, Germany) collagen-coated 25 cm^2 flasks in a humidified atmosphere containing 5 % CO_2 in air at 37 °C. The CFF-16HBEge cells were cultured in minimum essential medium (MEM) (Gibco, Life Technologies, Monza, Italy) supplemented with 10 % fetal bovine serum (Gibco, Life Technologies), 1 % l-glutamine, 1 % non-essential amino acids, and 1 % sodium pyruvate. Cell dissociation was performed every 3 days using TrypLE Express (Gibco, Life Technologies), and cells were seeded at a 1:3 ratio for maintenance in 25 cm^2 flasks.

2.3. Cell transfection

About 2×10^4 cells were seeded in 8-well Ibidi™ chamber slides, while 2×10^5 were seeded in 6-well plates the day before transfection. Lipofectamine® RNAiMAX reagent (Life Technologies, Monza, Italy) was used to transfect cells with the ASOs following manufacturer's instructions. 10 or 15 pmol of ASOs were transfected into 2×10^4 cells in

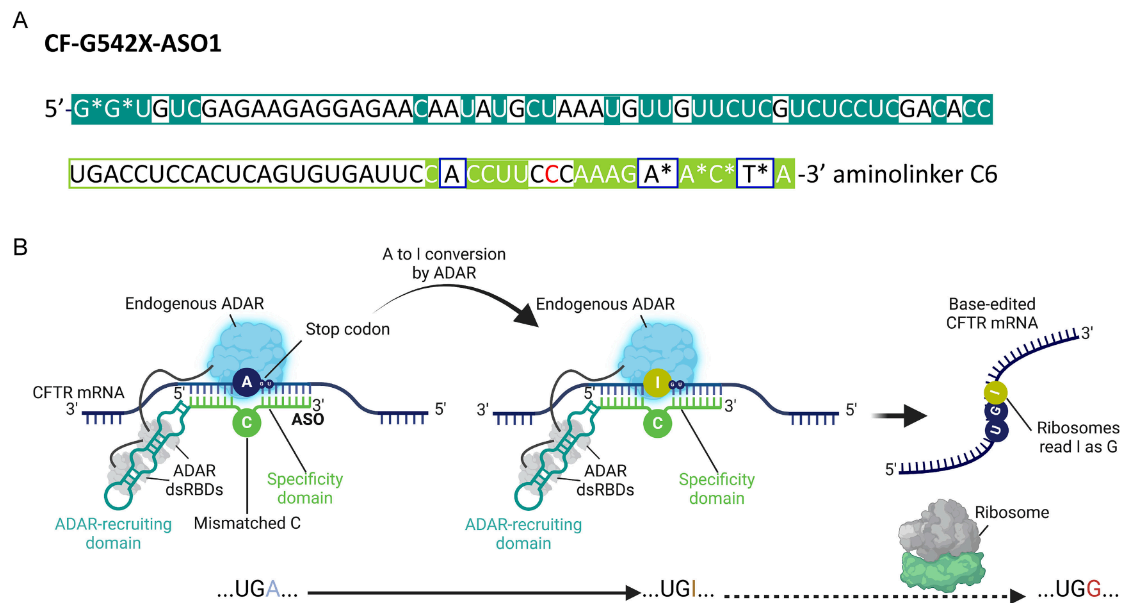

Fig. 1. The RESTORE strategy to rescue CFTR G542X mutation. (A) Antisense oligonucleotide (ASO) sequence for G542X mutation. In dark green is the ADAR-recruiting domain, in light green is the CFTR specific region, in red the nucleotide mismatch, RNA nucleotides have a white background, RNA nucleotides modified with 2′-O-Me have a green background, LNA are within blue squares, and nucleotides modified with phosphorothioates bonds are marked with an asterisk N*. (B) Schematic representation of the RESTORE system. It consists of a guide RNA alone, which recruits endogenous ADAR to edit the target adenosine within the CFTR mRNA sequence (Merkle et al., 2019). In dark green is the Adar-recruiting domain, in light green is the CFTR specific region with the mismatched C. Created in BioRender. Barra, V. (2024) https://BioRender.com/s34o632.

the ibidi™ chamber slides, and 100 or 150 pmol were transfected into 2 × 10^5 cells in the 6-well plates. After 24 or 48 hr from administration, the cells were harvested or fixed for further analysis.

2.4. Immunofluorescence

16HBE^{G542X} cells were seeded on PureCol coated-8-wells chamber (Ibidi™). After 24 or 48 hr of transfection, cells were fixed with 4 % paraformaldehyde (PFA) on ice for 10 min and then washed three times with 1X PBS. PFA was halted by a 15 min incubation with 1 mM glycine. Subsequently, cells were incubated for 10 min at 37 °C with Alexa Fluor 594 Wheat Germ Agglutinin (WGA) to label cell membranes (Thermo Fisher Scientific, Monza, Italy). Additionally, cells were permeabilized with 1 % Triton X-100 for 1.5 min and then blocked for 30 min at room temperature with 3 % BSA containing 0.1 % Triton X-100. Cells were incubated overnight at 4 °C with the primary antibody 570 which labels the cytosolic R domain of CFTR (provided by the Cystic Fibrosis Foundation at University of North Carolina), diluted 1:400, on constant oscillation. Afterwards, a secondary antibody, anti-mouse Cy-2 conjugated (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) was used, diluted 1:1,000 for 120 min using at room temperature on constant oscillation. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) and after 3 washes, the 8-wells chambers were covered with mounting media (90 % glycerol with pH adjusted to 8) before observation at 63x magnification on a Zeiss Axioskop microscope (Oberkochen, Germany) equipped for epifluorescence. ImageJ/Fiji analysis was performed by counting only the cells in which the CFTR protein was present on cell membranes. For each experimental replicate, two wells were used, and 10 images were acquired per well, analyzing at least 100 nuclei. Colocalization analysis was performed using the Colocalization Threshold plugin in ImageJ software, using the Pearson's correlation coefficient values.

2.5. RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA from transfected cells was extracted using the RNeasy Mini or Micro Kit (Qiagen, Milan, Italy), depending on the number of cells according to the manufacturer's instructions, and reverse transcribed with the High-capacity cDNA reverse transcription kit (Applied Biosystems™, Thermo Fisher Scientific, Monza, Italy). RT-qPCR was then performed as previously described (Veneziano et al., 2016). Briefly, cDNAs (75 ng/replicate) were amplified with SYBR™ Green master mix (Applied Biosystems, Life Technologies, Foster City, CA) supplemented with forward and reverse primers (1 μM each) indicated in the table below. CFTR primers were designed to amplify the region between the end of exon 24 and the beginning of exon 25 in order to evaluate the quantity of complete CFTR transcripts. Samples were analysed with the thermocycler Applied Biosystems 7300 Real Time PCR System (Thermo Fisher Scientific) 96-well thermocycler (15 s at 95 °C, 60 s at 60 °C, for 40 cycles).

2.6. Next-generation sequencing

cDNA was obtained from 16HBE^{G542X} cells treated with 15 pmol of ASOs for 24 hr and a fragment of about 1 kb, surrounding the position of the mutation (c.1624G > T), was amplified with primers CF forward (fw) and reverse (rev) (indicated in the table below) and sent to Bio-Fab Research (Rome, Italy) for analysis.

2.7. Oligonucleotides used in this study

NCBI sequence accession numbers:
GAPDH mRNA ID NM_002046.7.
GAPDH gene ID NG_007073.2.
CFTR mRNA ID NM_000492.4.
CFTR gene ID NG_016465.4.

		Gene location	mRNA location
GAPDH fw (Barra et al., 2022)	5′ tgacatcaagaaggtggtga 3′	nt 8336–8355	nt 844–863
GAPDH rev (Barra et al., 2022)	5′ tccaccaccctgttgctgta 3′	nt 8649–8630	nt 1053–1034
CFTR fw exon 24	5′ atccctatgaacagtggag 3′	nt 192117–192099	ex24 nt 3984–4002
CFTR rev exon 25	5′ ttaggacacagccccatc 3′	nt 203980–203962	nt 4109–4091
Next generation sequencing			
CF fw exons 9/10	5′ tgggaggagggatttgggga 3′	nt 87868–81325	nt 1271–1290
Cf rev exon 14	5′ acgctgatgcgaggcagtat 3′	nt 131645–131626	nt 2331–2312
ASO (CFTR specific region)			
CF-G542X exon 12	5′ugaccuccacucagugugauuccaccuucccaaagaacta 3′	nt 127026–126987	nt 1725–1686

3. Results

3.1. Antisense RNA oligonucleotide design

We designed ASOs to target endogenous ADAR to the adenine in positions 1,626 in the CFTR cDNA sequence to recode the premature termination codon G542X from nonsense to coding (UGA → UGG) (Fig. 1). ASOs are composed of 95 nucleotides. The 3′ programmable end is a sequence of 40 nucleotides complementary to the specific region of the CFTR mRNA, where the PTC is located, with a mismatched cytidine opposite the target adenosine in position 11 from the 3′ terminal nucleotide. The invariant 5′ region of 55 nucleotides form the 25 bp loop recognized by ADARs (Merkle et al., 2019). To improve stability and binding affinity, we introduced different modifications based on the ASO design, as previously reported by Merkel and colleagues (Merkle et al., 2019). In particular, the first three 5′ nucleotides are linked by PS bonds, as well as the last five nucleotides at the 3′ end, with the aim of increasing resistance to exonucleases and cellular uptake (Putney et al., 1981). Furthermore, multiple ribonucleotides of the sequence were modified by 2′-*O*-methylation, as this modification enhances the stability and cellular uptake advantages already provided by the PS bonds (Mansoor and Melendez, 2008). Finally, two LNA bases were introduced in the sequence, in the region complementary to the target, since their constrained ring structure has been shown to improve ASO binding affinity (Wahlestedt et al., 2000).

3.2. CFTR protein rescue at the plasma membrane by using the RESTORE strategy in CFTR mutated cells

We investigated the RESTORE RNA editing strategy to correct the UGA premature stop codon in the CFTR mRNA of the bronchial

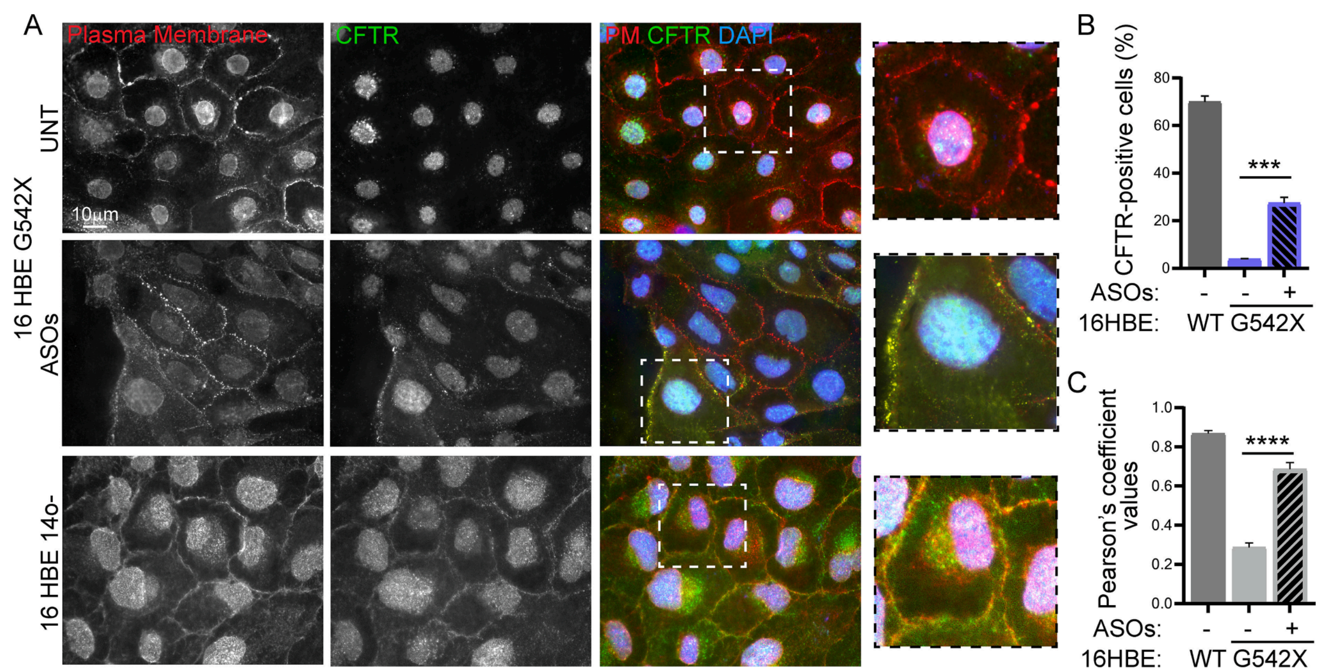

Fig. 2. The RESTORE strategy rescues CFTR protein at the plasma membrane in 16HBE^{G542X} mutated cells. (**A**) Representative fluorescence microscopy images depicting the colocalization (yellow) between the plasma membrane (red) and CFTR (green) in 16HBE^{G542X} cells transfected with ASOs or left untransfected (UNT). Nuclei (blue) were stained with DAPI. 16HBE14o- cell line was used as a control. Images were taken at 63x magnification on a Zeiss microscope equipped for epifluorescence. (**B**) Quantification of CFTR-positive cells in the IF for 16HBE^{G542X} cells after transfection with ASOs. Only cells showing CFTR signal (green) on the plasma membrane (red), were considered positive. The graph shows the mean of four independent experiments. The error bars represent the standard error of the mean (SEM). Unpaired *t*-test between the UNT sample and the antisense RNA oligonucleotide treatment: *** $p \leq 0.001$. (**C**) Pearson's colocalization coefficient analysis between the plasma membrane (PM) and the CFTR protein. Unpaired *t*-test between the UNT sample and the ASOs treatment: **** $p \leq 0.0001$.

epithelial cell line 16HBEG542X (GGA > TGA). The editing of this mutation would not rescue the original amino acid but introduce a tryptophan (UGG), enabling a limited CFTR activity (Xue et al., 2017). In order to apply the RESTORE platform, 16HBE^{G542X} cells were transfected with different amounts (10 and 15 pmol/well) of the designed ASOs to test their efficiency at either 24 or 48 hr post-transfection. The immunofluorescence (IF) for CFTR was used to evaluate CFTR rescue on the plasma membrane (Figs. S1A, B). The optimal condition (15 pmol ASOs for 24 h) used for further analysis, is shown in Fig. 2. In Fig. 2A, there are representative IF images that demonstrate the restoration of CFTR protein on the plasma membrane in ASO-transfected 16HBE^{G542X} mutant cell line compared to the untransfected mutated cells (UNT). We also quantified the percentage of CFTR-positive cells, defined as those with CFTR colocalizing with an antibody against glycosylated proteins of the plasma membrane, indicating the proper processing and localization of CFTR protein, which was observed in 28 % of the 16HBE^{G542X} cells (Fig. 2B). The colocalization is confirmed by the Pearson's coefficient analysis which is approximately 0.7 compared to the 0.8–0.9 observed in 16HBE14o- cells, denoting a positive correlation between the plasma membrane and the CFTR protein (Fig. 2C). Although we mainly focused on the G542X mutation, we conducted preliminary tests to assess whether the system could also be effective for other types of nonsense mutations. Specifically, we designed and tested ASOs targeting the adenine at position 3,846 in the W1282X (c.3846G > A) mutation, hereafter referred to as 16HBE^{W1282X} (Fig. S2A). This approach successfully showed the localization of CFTR protein to the plasma membrane in 16HBE^{W1282X} cells (Figs. S2B, C) with a positive Pearson's correlation (0.6–0.7) (Fig. S2D).

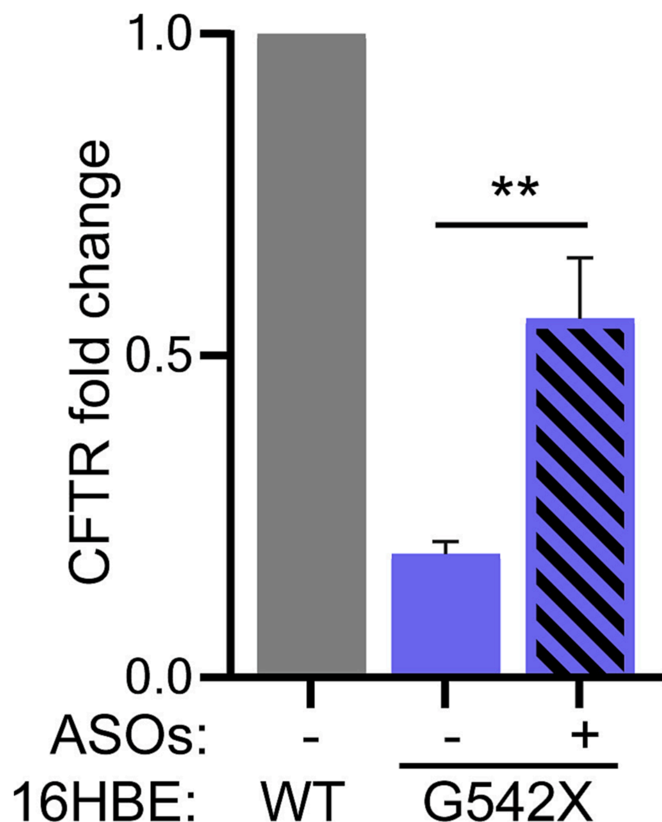

Fig. 3. Recover of full-length CFTR transcript after RNA editing. RT-qPCR histograms show the increased amount of CFTR mRNA in 16HBE^{G542X} cells after treatment with ASOs with respect to the 16HBE14o- cells. The graphs show the mean of four replicates. The error bars represent the SEM. Unpaired *t*-test between the UNT sample and the ASOs treatment: ** $p \leq 0.05$.

Reference WT sequence	A	C	T	G
1619>T	0.0218	0.0459	99.9127	0.0153
1620>T	0.0547	0.0219	99.8798	0.0394
1621>C	0.0349	99.9411	0.0153	0.0044
1622>T	0.0175	0.0547	99.8161	0.1073
1623>T	0.0285	0.0154	99.9188	0.0329
1624>G	0.0090	0.0291	99.8722 *	0.0830
1625>G	0.0280	0.0000	0.0216	99.9461
1626>A	98.5241	0.0215	0.0194	1.4286
1627>G	0.0085	0.0021	0.0340	99.9512
1628>A	99.9384	0.0212	0.0064	0.0149
1629>A	99.9192	0.0276	0.0170	0.0319

Fig. 4. RESTORE strategy edits the G542X mutation in the CFTR mRNA. Next generation sequencing confirms the mutation G > T at position 1,624 (indicated with an asterisk in the figure) specific for the G542X mutant, and the targeted editing of CFTR mRNA. NGS results of ASO-treated 16HBE^{G542X} cells showed 1,4% of editing of adenosine at position 1,626 in the CFTR cDNA.

3.3. CFTR mRNA increase following antisense RNA oligonucleotide transfection in CFTR mutated cells

Next, we evaluated the amount of CFTR transcripts by RT-qPCR to assess the efficacy of the RESTORE system in increasing the expression of CFTR transcript carrying the edited adenine at the PTCs. The results shown in Fig. 3 demonstrate that the amount of the CFTR transcript in 16HBE^{G542X} cells treated with ASOs was increased of 3.5-fold in comparison to the expression level of the untreated cells. A similar effect was observed in preliminary tests with 16HBE^{W1282X} cells treated with ASOs (Fig. S2E). To test if ASOs allowed the editing of the PTC codon, we sequenced CFTR cDNA of transfected 16HBE^{G542X} cells by next generation sequencing (NGS, BIO-Fab Research, Rome, Italy). In the G542X CFTR mRNA, we observed a 1.4 % guanosine instead of adenosine (nucleotide position 1,626) within the nonsense codon. This percentage is significantly higher if compared to the presence of guanosine in other positions where it was not expected, confirming the mRNA editing at the intended site (Fig. 4).

4. Discussion

Currently, there is no Food and Drug Administration (FDA)-approved therapy for people with CF carrying nonsense mutations. Some molecules, including translational readthrough inducing drugs (TRIDs), have been reported to correct stop mutations by introducing a near-cognate

amino acid into the nascent protein at the PTC (Sharma et al., 2021) but they are still under investigation. Thus, we still need to explore new strategies for a specific and successful treatment against stop mutations. In this context, gene therapy approaches are considered potential alleys in correcting the mutation in the genome. In addition to genome editing, RNA editing strategies offer unique opportunities to address CFTR defects at the RNA level without altering the DNA that would be as permanent as the eventual side effects. Here, we took advantage of the activity of the endogenous ADAR enzymes to deaminate the adenosine of the premature termination codon into inosine that will be read as guanosine by the ribosome. We designed specific ASOs able to recruit endogenous ADARs to the target site in the CFTR mRNA. In principle, the targeted activity of the endogenous ADARs would change the UGA nonsense codon into a UGG tryptophan codon, thus restoring the full translation of the CFTR protein (Xue et al., 2017). In addition, the invariant domain of the ASOs we used, and their chemical modifications have been observed to preferentially induce editing by the ADAR1 p150 isoform (Merkle et al., 2019), which was demonstrated to allow a very efficient RNA editing in the cytoplasm (Wong et al., 2003). This could indicate that in our system, editing of the CFTR mRNA takes place in the cytoplasm. Our results suggest the effectiveness of this specific RNA editing tool in correcting the premature stop codon in the CFTR transcript of isogenic CFF-16HBEge human bronchial cells carrying the CFTR G542X stop mutation. Our results indicate an increase in the percentage of CFTR-positive cells as determined by IF assays in both CFTR mutant cell lines. Even though the number of CFTR-positive cells appeared low, it is similar to the one observed in the cells treated with G418 (Chiavetta et al., 2023), a well-known TRID. It is also important to note that we considered as positive only the cells where the CFTR protein localized to the plasma membrane by IF assay; this condition is present in 70 % of the 16HBE14o- cells in our experiments. Technical limitations or the absence of apical-basal polarity in the culture condition could affect the correct esteem of CFTR recovery. Unfortunately, we were not able to detect the rescue of the CFTR protein after RNA-editing by western blotting (data not shown), which might suggest a rescue below the sensitivity of this technique. And yet, we assessed an increased expression level of the CFTR transcript at exons 24 and 25 after the transfection of the ASOs by RT-qPCR, supporting the IF results. NGS data revealed that 1.4 % of the CFTR transcripts had been edited, suggesting that the observed increase of CFTR transcription could stem from the stabilization of CFTR mutated RNA due to the ASOs. This indicates that endogenous ADARs can effectively mediate relevant changes in RNA, even if the overall percentage of edited RNA is low. We did not investigate further on this, but we compared our results with those reported in the current literature where similar systems of endogenous ADAR-based RNA editing were used. Merkle and colleagues used the RESTORE strategy to prove its therapeutic potential for two mutated disease genes, STAT1 and SERPINA1. In both cases they showed editing yields of 7–21 % and 10 %, respectively (Merkle et al., 2019), which are higher than the ones we obtained for CFTR. However, it is to consider that in both cases studied by Merkle and colleagues the genes were highly expressed, meaning that the amount of editable RNA is high. Instead, in our case, editable CFTR RNA is present at very low levels, which could explain the reduced efficiency. Similarly, Katrekar and colleagues, by using a very similar approach that recruits endogenous ADARs to the target RNA, showed that in an in vivo mouse model for Duchenne muscular dystrophy, the editing yield of dystrophin was 2.4–3.6 % depending on the type of mutation (Katrekar et al., 2019). However, even with this low percentage of editing, IF assay revealed clear restoration of dystrophin expression and nitric oxide synthase (nNOS) activity at the sarcolemma. These in vivo results are in line with the apparently contrasting data we obtained for CFTR restoration. We hypothesize that, although low, the amount of edited CFTR mRNA is sufficient to recover the full-length CFTR we observed by IF on the plasma membrane, even though it remains to be determined if the CFTR channel function was restored as well. To this regard, the missense mutation G542W that is introduced by the editing should be less severe that the nonsense mutation itself, as reported by Premchandar and colleagues that showed how the functional expression defect of the G542W mutant can be significantly rescued by treatment with corrector combinations 3C (VX-809 + 3151 + 4172) or 2C (VX-661 + VX-445) (Premchandar et al., 2024).

We believe that sequence-specific RNA editing strategies that employ ASOs to edit the PTC provides valuable insights for the development of innovative therapies aimed to correct nonsense mutations in CF patients with stop mutations who cannot benefit from current therapies. Moreover, combining the RNA editing tools with other molecules that can preserve the mRNA harbouring the PTC from degradation for example, by inhibiting the nonsense mediated decay (NMD) pathway might lead to better results. Also, by correcting CFTR mRNA, RNA editing appears to be safer than genome editing whose eventual side effects would be permanent, and provides a more flexible and adaptable approach for CF therapy. Despite, several challenges still remain, including delivery systems and safety considerations, advancements and refinement of RNA editing techniques hold the potential to address the consequences of the genetic mutation, offering a revolutionary approach to treatment for CF patients who currently lack effective therapies.

CRediT authorship contribution statement

Simona Titoli: Writing – review & editing, Writing – original draft, Investigation. **Viviana Barra:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis. **Serena Gargano:** Investigation. **Aldo Di Leonardo:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Raffaella Melfi:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Aquino-Jarquin, G., 2020. Novel engineered programmable systems for ADAR-mediated RNA editing. Mol. Ther. Nucleic Acids 19, 1065–1072. https://doi.org/10.1016/j.omtn.2019.12.042.

Barra, V., Chiavetta, R.F., Titoli, S., Provenzano, I.M., Carollo, P.S., Di Leonardo, A., 2022. Specific irreversible cell-cycle arrest and depletion of cancer cells obtained by combining curcumin and the flavonoids quercetin and fisetin. Genes 13, 1125. https://doi.org/10.3390/genes13071125.

Bellingrath, J.-S., McClements, M.E., Fischer, M.D., MacLaren, R.E., 2023. Programmable RNA editing with endogenous ADAR enzymes - a feasible option for the treatment of

inherited retinal disease? Front. Mol. Neurosci. 16, 1092913. https://doi.org/10.3389/fnmol.2023.1092913.

Booth, B.J., Nourreddine, S., Katrekar, D., Savva, Y., Bose, D., Long, T.J., Huss, D.J., Mali, P., 2023. RNA editing: Expanding the potential of RNA therapeutics. Mol. Ther. 31, 1533–1549. https://doi.org/10.1016/j.ymthe.2023.01.005.

Carollo, P.S., Tutone, M., Culletta, G., Fiduccia, I., Corrao, F., Pibiri, I., Di Leonardo, A., Zizzo, M.G., Melfi, R., Pace, A., Almerico, A.M., Lentini, L., 2023. Investigating the Inhibition of FTSJ1, a Tryptophan tRNA-Specific 2′-O-Methyltransferase by NV TRIDs, as a Mechanism of Readthrough in Nonsense Mutated CFTR. Int. J. Mol. Sci. 24, 9609. https://doi.org/10.3390/ijms24119609.

Chiavetta, R.F., Titoli, S., Barra, V., Cancemi, P., Melfi, R., Leonardo, A.D., 2023. Site-Specific RNA Editing of Stop Mutations in the CFTR mRNA of Human Bronchial Cultured Cells. Int. J. Mol. Sci. 24. https://doi.org/10.3390/ijms241310940.

Corrao, F., Zizzo, M.G., Tutone, M., Melfi, R., Fiduccia, I., Carollo, P.S., Leonardo, A.D., Caldara, G., Perriera, R., Pace, A., Belmonte, B., Sammataro, S., Pibiri, I., Lentini, L., 2022. Nonsense codons suppression. An acute toxicity study of three optimized TRIDs in murine model, safety and tolerability evaluation. Biomed. Pharmacother. 156, 113886. https://doi.org/10.1016/j.biopha.2022.113886.

Cox, D.B.T., Gootenberg, J.S., Abudayyeh, O.O., Franklin, B., Kellner, M.J., Joung, J., Zhang, F., 2017. RNA editing with CRISPR-Cas13. Science 358, 1019–1027. https://doi.org/10.1126/science.aaq0180.

Cystic Fibrosis Mutation Database URL http://genet.sickkids.on.ca/ (accessed 2.9.24).

Gold, A., Levanon, E.Y., Eisenberg, E., 2021. The New RNA-Editing Era – Ethical Considerations. Trends Genet. 37, 685–687. https://doi.org/10.1016/j.tig.2021.04.013.

Han, W., Huang, W., Wei, T., Ye, Y., Mao, M., Wang, Z., 2022. Programmable RNA base editing with a single gRNA-free enzyme. Nucleic Acids Res. 50, 9580–9595. https://doi.org/10.1093/nar/gkac713.

Harrison, P.T., 2022. CFTR RNA- and DNA-based therapies. Curr. Opin. Pharmacol. 65, 102247. https://doi.org/10.1016/j.coph.2022.102247.

Katrekar, D., Chen, G., Meluzzi, D., Ganesh, A., Worlikar, A., Shih, Y.-R., Varghese, S., Mali, P., 2019. In vivo RNA editing of point mutations via RNA-guided adenosine deaminases. Nat. Methods 16, 239–242. https://doi.org/10.1038/s41592-019-0323-0.

Lentini, L., Melfi, R., Leonardo, A.D., Spinello, A., Barone, G., Pace, A., Piccionello, A.P., Pibiri, I., 2014. Toward a Rationale for the PTC124 (Ataluren) Promoted Readthrough of Premature Stop Codons: A Computational Approach and GFP-Reporter Cell-Based Assay. Mol. Pharm. 11, 653. https://doi.org/10.1021/mp400230s.

Mansoor, M., Melendez, A.J., 2008. Advances in antisense oligonucleotide development for target identification, validation, and as novel therapeutics. Gene Regul. Syst. Biol. 2, GRSB.S418. https://doi.org/10.4137/GRSB.S418.

Marina, R.J., Brannan, K.W., Dong, K.D., Yee, B.A., Yeo, G.W., 2020. Evaluation of engineered CRISPR-Cas-mediated systems for site-specific RNA editing. Cell Rep. 33. https://doi.org/10.1016/j.celrep.2020.108350.

Melfi, R., Cancemi, P., Chiavetta, R., Barra, V., Lentini, L., Di Leonardo, A., 2020. Investigating REPAIRv2 as a Tool to Edit CFTR mRNA with Premature Stop Codons. Int. J. Mol. Sci. 21, 4781. https://doi.org/10.3390/ijms21134781.

Merkle, T., Merz, S., Reautschnig, P., Blaha, A., Li, Q., Vogel, P., Wettengel, J., Li, J.B., Stafforst, T., 2019. Precise RNA editing by recruiting endogenous ADARs with antisense oligonucleotides. Nat. Biotechnol. 37, 133–138. https://doi.org/10.1038/s41587-019-0013-6.

Montiel-Gonzalez, M.F., Vallecillo-Viejo, I., Yudowski, G.A., Rosenthal, J.J.C., 2013. Correction of mutations within the cystic fibrosis transmembrane conductance regulator by site-directed RNA editing. Proc. Natl. Acad. Sci. u. s. a. 110, 18285–18290. https://doi.org/10.1073/pnas.1306243110.

Nishikura, K., 2016. A-to-I editing of coding and non-coding RNAs by ADARs. Nat. Rev. Mol. Cell Biol. 17, 83–96. https://doi.org/10.1038/nrm.2015.4.

Patterson, J.B., Samuel, C.E., 1995. Expression and regulation by interferon of a double-stranded-RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. Mol. Cell. Biol. 15, 5376–5388.

Perriera, R., Vitale, E., Pibiri, I., Carollo, P.S., Ricci, D., Corrao, F., Fiduccia, I., Melfi, R., Zizzo, M.G., Tutone, M., Pace, A., Lentini, L., 2023. Readthrough Approach Using NV Translational Readthrough-Inducing Drugs (TRIDs): A Study of the Possible Off-Target Effects on Natural Termination Codons (NTCs) on TP53 and Housekeeping Gene Expression. Int. J. Mol. Sci. 24, 15084. https://doi.org/10.3390/ijms242015084.

de Poel, E., Spelier, S., Suen, S.W.F., Kruisselbrink, E., Graeber, S.Y., Mall, M.A., Weersink, E.J.M., van der Eerden, M.M., Koppelman, G.H., van der Ent, C.K., Beekman, J.M., 2022. Functional Restoration of CFTR Nonsense Mutations in Intestinal Organoids. J. Cyst. Fibros. 21, 246–253. https://doi.org/10.1016/j.jcf.2021.09.020.

Pranke, I., Golec, A., Hinzpeter, A., Edelman, A., Sermet-Gaudelus, I., 2019. Emerging Therapeutic Approaches for Cystic Fibrosis. From Gene Editing to Personalized Medicine. Front. Pharmacol. 10, 121. https://doi.org/10.3389/fphar.2019.00121.

Premchandar, A., Ming, R., Baiad, A., Da Fonte, D.F., Xu, H., Faubert, D., Veit, G., Lukacs, G.L., 2024. Readthrough-induced misincorporated amino acid ratios guide mutant-specific therapeutic approaches for two CFTR nonsense mutations. Front. Pharmacol. 15. https://doi.org/10.3389/fphar.2024.1389586.

Putney, S.D., Benkovic, S.J., Schimmel, P.R., 1981. A DNA fragment with an alpha-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated in vivo. Proc. Natl. Acad. Sci. 78, 7350–7354. https://doi.org/10.1073/pnas.78.12.7350.

Quemener, A.M., Bachelot, L., Forestier, A., Donnou-Fournet, E., Gilot, D., Galibert, M.-D., 2020. The powerful world of antisense oligonucleotides: from bench to bedside. Wiley Interdiscip. Rev. RNA 11, e1594.

Rang, C., Keating, D., Wilson, J., Kotsimbos, T., 2020. Re-imagining cystic fibrosis care: next generation thinking. Eur. Respir. J. 55. https://doi.org/10.1183/13993003.02443-2019.

Regard, L., Martin, C., Da Silva, J., Burgel, P.-R., 2023. CFTR Modulators: current status and evolving knowledge. Semin. Respir. Crit. Care Med. 44, 186–195. https://doi.org/10.1055/s-0042-1758851.

Sharma, J., Du, M., Wong, E., Mutyam, V., Li, Y., Chen, J., Wangen, J., Thrasher, K., Fu, L., Peng, N., Tang, L., Liu, K., Mathew, B., Bostwick, R.J., Augelli-Szafran, C.E., Bihler, H., Liang, F., Mahiou, J., Saltz, J., Rab, A., Hong, J., Sorscher, E.J., Mendenhall, E.M., Coppola, C.J., Keeling, K.M., Green, R., Mense, M., Suto, M.J., Rowe, S.M., Bedwell, D.M., 2021. A small molecule that induces translational readthrough of CFTR nonsense mutations by eRF1 depletion. Nat. Commun. 12, 4358. https://doi.org/10.1038/s41467-021-24575-x.

Valley, H.C., Bukis, K.M., Bell, A., Cheng, Y., Wong, E., Jordan, N.J., Allaire, N.E., Sivachenko, A., Liang, F., Bihler, H., Thomas, P.J., Mahiou, J., Mense, M., 2019. Isogenic cell models of cystic fibrosis-causing variants in natively expressing pulmonary epithelial cells. J. Cyst. Fibros. 18, 476–483. https://doi.org/10.1016/j.jcf.2018.12.001.

Veneziano, L., Barra, V., Lentini, L., Spatafora, S., Di Leonardo, A., 2016. p14ARF prevents proliferation of aneuploid cells by inducing p53-dependent apoptosis. J. Cell. Physiol. 231, 336–344. https://doi.org/10.1002/jcp.24976.

Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hökfelt, T., Broberger, C., Porreca, F., Lai, J., Ren, K., Ossipov, M., Koshkin, A., Jakobsen, N., Skouv, J., Oerum, H., Jacobsen, M.H., Wengel, J., 2000. Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. Proc. Natl. Acad. Sci. 97, 5633–5638. https://doi.org/10.1073/pnas.97.10.5633.

Wang, G., 2023. Genome editing for cystic fibrosis. Cells 12, 1555. https://doi.org/10.3390/cells12121555.

Wong, S.K., Sato, S., Lazinski, D.W., 2003. Elevated activity of the large form of ADAR1 in vivo: very efficient RNA editing occurs in the cytoplasm. RNA 9, 586–598. https://doi.org/10.1261/rna.5160403.

Xu, C., Zhou, Y., Xiao, Q., He, B., Geng, G., Wang, Z., Cao, B., Dong, X., Bai, W., Wang, Y., Wang, X., Zhou, D., Yuan, T., Huo, X., Lai, J., Yang, H., 2021. Programmable RNA editing with compact CRISPR-Cas13 systems from uncultivated microbes. Nat. Methods 18, 499–506. https://doi.org/10.1038/s41592-021-01124-4.

Xue, X., Mutyam, V., Thakerar, A., Mobley, J., Bridges, R.J., Rowe, S.M., Keeling, K.M., Bedwell, D.M., 2017. Identification of the amino acids inserted during suppression of CFTR nonsense mutations and determination of their functional consequences. Hum. Mol. Genet. 26, 3116. https://doi.org/10.1093/hmg/ddx196.