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Short Communication

# Expression of the inflammatory regulator A20 correlates with lung function in patients with cystic fibrosis

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## Abstract

**Background:** A20 and TAX1BP1 interact to negatively regulate NF- $\kappa$ B-driven inflammation. A20 expression is altered in F508del/F508del patients. Here we explore the effect of CFTR and CFTR genotype on A20 and TAX1BP1 expression. The relationship with lung function is also assessed.

**Methods:** Primary nasal epithelial cells (NECs) from CF patients (F508del/F508del,  $n=7$ , R117H/F508del,  $n=6$ ) and controls (age-matched,  $n=8$ ), and 16HBE14o- cells were investigated. A20 and TAX1BP1 gene expression was determined by qPCR.

**Results:** Silencing of CFTR reduced basal A20 expression. Following LPS stimulation A20 and TAX1BP1 expression was induced in control NECs and reduced in CF NECs, broadly reflecting the CF genotype: F508del/F508del had lower expression than R117H/F508del. A20, but not TAX1BP1 expression, was proportional to FEV<sub>1</sub> in all CF patients ( $r=0.968$ ,  $p<0.001$ ).

**Conclusions:** A20 expression is reduced in CF and is proportional to FEV<sub>1</sub>. Pending confirmation in a larger study, A20 may prove a novel predictor of CF inflammation/disease severity.

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**Keywords:** A20 protein; FEV<sub>1</sub>; NF kappa B; Airway epithelial cells; Cystic Fibrosis; Chronic inflammation

## 1. Introduction

Cystic Fibrosis (CF) lung disease is heterogeneous in nature and the severity of disease in patients with the same genotype can vary significantly. Activation of TLR4 by Gram-negative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) leads to pro-longed NF- $\kappa$ B (p65) signalling in CF primary epithelial cells and CF cell lines [1,2]. A20 (*TNFAIP3*, tumor necrosis factor, alpha-induced protein 3) is an endogenous negative regulator of the NF- $\kappa$ B pathway and is rapidly and transiently induced in response to bacterial and viral stimuli. A20 terminates NF- $\kappa$ B driven inflammation by inhibiting the

polyubiquitination and activation of the central adaptor protein TNF receptor-associated factor (TRAF) 6 [3]. Desensitized pathogen recognition is thought to be secondary to hyperactive TRAF6 signalling in chronic lung diseases including CF [4].

Despite varied pathogenesis, A20 is a critical regulator of inflammatory processes in rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, multiple sclerosis, psoriasis and inflammatory bowel disease [5]. Furthermore, A20 is a susceptibility gene for the development of Crohn's [6] and irritable bowel disease [7]. In the lung, *P. aeruginosa* challenge rapidly induces A20 in mice [8], while A20 is essential for termination of TLR2/4 mediated IL-8 release from primary airway epithelial cells [9]. We recently showed that A20 is reduced in CF airway epithelial cells after stimulation with LPS. The action of A20 is dependent on an adapter protein called TAX1BP1, which is also significantly reduced in LPS-treated CF cells [1,10]. Furthermore, this work suggested that CF airway epithelial cells lack

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essential interactions between A20, TAX1BP1 and TRAF6, which may partly explain the associated chronic activation of NF- $\kappa$ B [1].

Here we further investigate the relationship between A20, TAX1BP1 and CFTR as well as any association with lung function (FEV<sub>1</sub>) in patients homozygous for F508del and heterozygous for R117H/F508del.

## 2. Materials and methods

Primary nasal epithelial cells (NEC) were obtained from CF patients (F508del/F508del {*n*=7}, R117H/F508del {*n*=6}) and age matched controls {*n*=8} as previously described [11] and were fully differentiated at air–liquid interface (ALI). NECs were treated with LPS (*P. aeruginosa*, Sigma, 50  $\mu$ g/ml) for 24 h. The

Research Ethics Committee of Northern Ireland approved the study and all participants provided informed consent (07/NIR02/23). In CF patients lung function (FEV<sub>1</sub>) was also determined at time of cell sampling.

The 16HBE14o- bronchial epithelial cell line (D. Gruenert UCSF, USA) was grown in submersion and transfected at 80% confluence with commercially available CFTR siRNA (GenomeWide siRNA, Qiagen) and RNAiFect™ Transfection Reagent (Qiagen). Experiments included mock (transfection reagent only) and scrambled (Allstars Neg siRNA, Qiagen) controls. Transfection efficiency was confirmed by qPCR as 72% $\pm$ 5.34%.

Total RNA was extracted using an RNeasy kit (Qiagen) and quantified on a Nanodrop (Thermo Scientific). Equal amounts of RNA were reverse transcribed into cDNA (Sensiscript Reverse

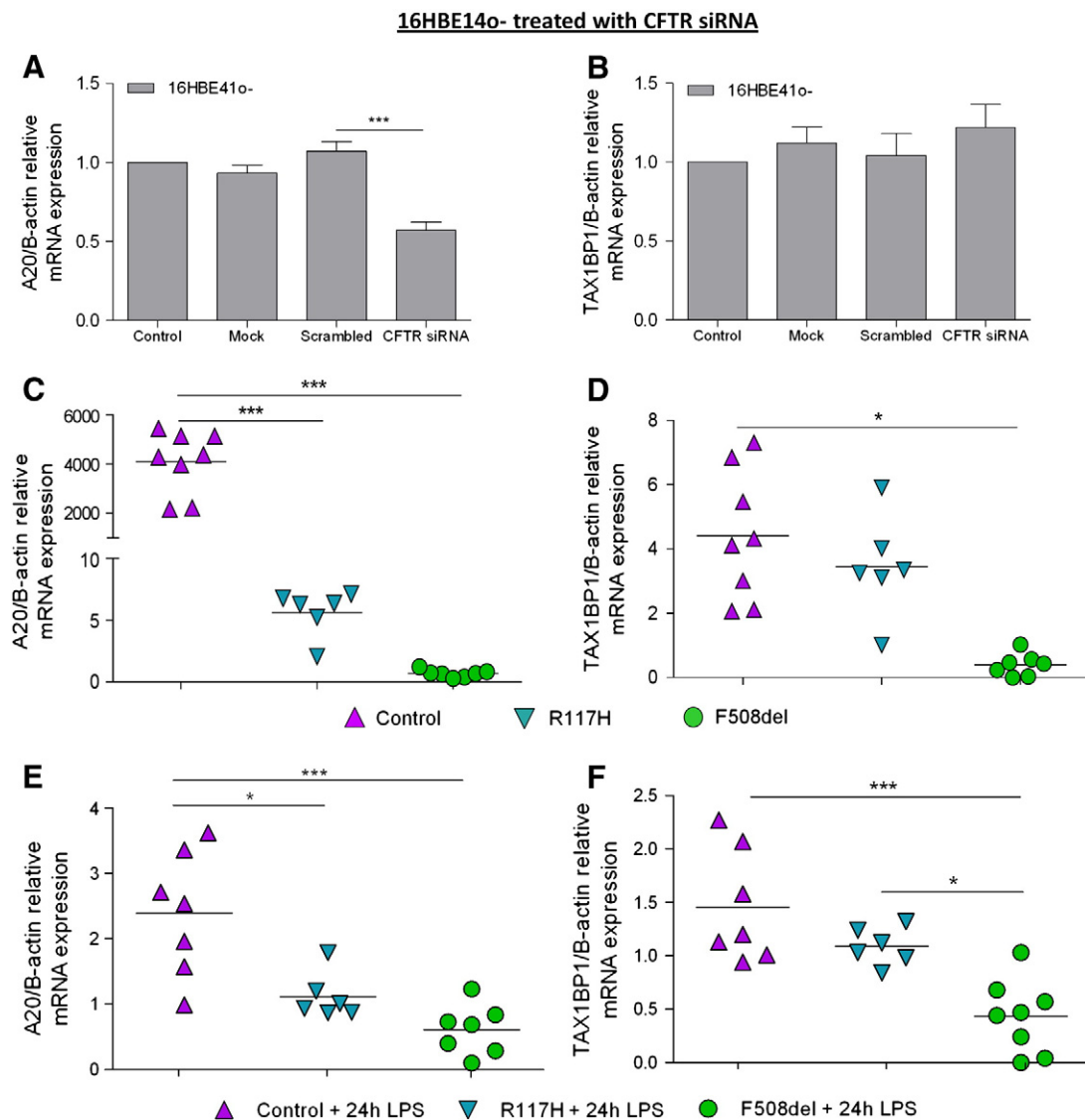


Fig. 1. The effect of CFTR and genotype on A20 and TAX1BP1 mRNA expression. The basal expression of A20 (A) and TAX1BP1 (B) was assessed by qPCR in 16HBE14o- cells treated with siRNA against CFTR. Data are presented as mean $\pm$ SEM with *n*=3. \*\*\**p*<0.001 compared with scrambled siRNA. The effect of different CF genotypes (R117H/F508del and F508del/F508del) on basal A20 (C) and TAX1BP1 (D) mRNA expression was determined by qPCR. Basal expression was expressed relative to Jurkat cells, which acted as an internal calibrator for all experiments. Furthermore, the mRNA expression of LPS-induced A20 (E) and TAX1BP1 (F) was also determined by qPCR. Expression was calculated after 24 h simulation with LPS and data are presented as mean $\pm$ SEM with *n*=6–8 as indicated in the figure. \**p*<0.05, \*\*\**p*<0.001 between groups as indicated in the figure.

Transcription Kit, Qiagen). A20, TAX1BP1 and p65 expression was assessed by qPCR [1]. Relative expression to  $\beta$ -actin was calculated using the  $\Delta\Delta$ Ct method. To overcome inter-patient variability in basal gene expression levels, mRNA expression 24 h after LPS stimulation was compared with mRNA expression at 0 h (standardized to 1) for each individual sample. cDNA obtained from Jurkat cells acted as an internal calibrator for all experiments and was used to determine differences in basal gene expression.

All data are presented as the means  $\pm$  SEM. Differences between groups were analysed using non-parametric tests and were considered to be significant if  $p < 0.05$ .

### 3. Results

CFTR silencing in 16HBE14o- cells caused a 43% reduction ( $p < 0.001$ ) in basal A20 expression, but did not alter basal TAX1BP1 levels (Fig. 1A and B). A reduction in basal A20 and TAX1BP1 expression was also confirmed in CF NECs compared

to control NECs (Fig. 1C and D). Following LPS stimulation, A20 was induced in control NECs and reduced in CF NECs. F508del homozygotes showed a more pronounced reduction in A20 expression than R117H/F508del heterozygotes ( $p < 0.05$ , Fig. 1E). However, a reduction in TAX1BP1 expression was only observed in F508del homozygotes ( $p < 0.05$ , Fig. 1F).

To provide a basic indication of how reduced A20 expression may relate to NF- $\kappa$ B and the inflammatory response we assessed the expression of p65 (NF- $\kappa$ B subunit) by qPCR. Following LPS stimulation, p65 expression was higher in F508del homozygotes than R117H/F508del heterozygotes ( $p < 0.05$ , Fig. 2A). Basal A20 expression was inversely proportional to p65 levels (Fig. 2B). A relationship between p65 and TAX1BP1 was not observed, but a clear separation of the two genotypes was observed (Fig. 2C).

We also assessed the relationship between basal A20 and TAX1BP1 mRNA expression and the FEV<sub>1</sub> of CF cohort (Fig. 2D and E). We only included CF patients who had also had FEV<sub>1</sub> measured on the same day as their nasal brushing sample was taken in this analysis. The median FEV<sub>1</sub> for the R117H/F508del

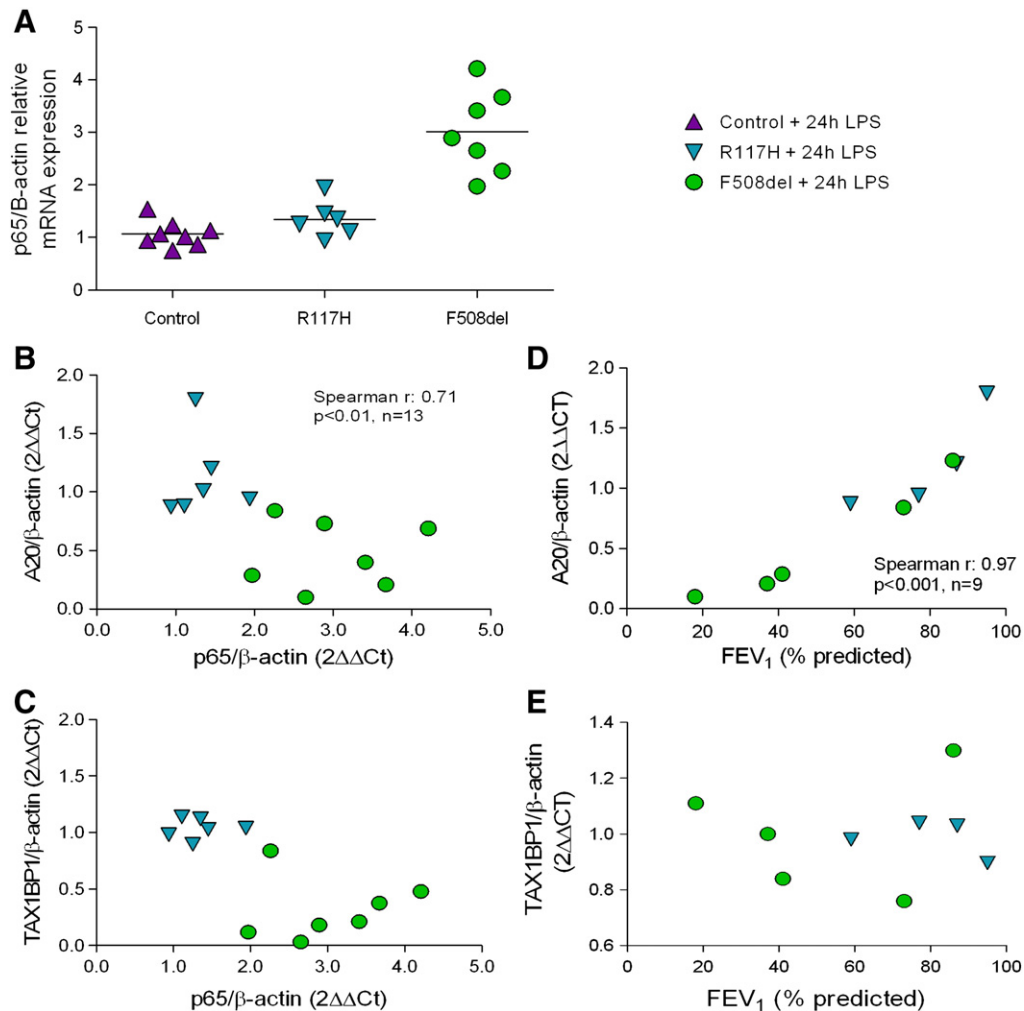


Fig. 2. Relationship between A20, TAX1BP1, p65 and lung function in different CF genotypes. The effect of different CF genotypes (R117H/F508del and F508del/F508del) on LPS-induced p65 (A) expression was determined by qPCR. Expression was calculated after 24 h stimulation with LPS and data are presented as mean  $\pm$  SEM with  $n = 6-8$  as indicated in the figure. \* $p < 0.05$ , \*\* $p < 0.01$  between groups as indicated in the figure. The relationship between p65 and A20 (B) or TAX1BP1 (C) expression was assessed by Spearman rank test. Significant correlations are indicated in the figure ( $r$  value). The relationship between FEV<sub>1</sub> and A20 (D) or TAX1BP1 (E) expression was assessed by Spearman rank test. Significant correlations are indicated in the figure ( $r$  value).

Table 1  
Patient demographics.

Patient demographics including FEV<sub>1</sub> are shown in Table 1.

	Group 1: Non-CF, healthy individual	Group 2: R117H/F508del CF patients	Group 3: F5808del /F508del CF patients
% Female	55	40	60
Median Age	27 (20, 29.5)	29 (28, 35)	25 (15, 35)
Median BMI	25.3 (22.4, 26.8)	24.5 (21.6, 27.5)	22.3 (20.7, 26.3)
FEV <sub>1</sub> (% predicted)	–	82.5 (70, 95.3)	50 (36, 62)
sputum for +ve <i>P.aeruginosa</i>	–	–	100%
Smokers	–	–	–

heterozygous and F508del homozygous groups were 82.5 and 50.05% predicted (Table 1), respectively, and FEV<sub>1</sub> was significantly correlated with A20 expression (Fig. 2D; Spearman rank test  $r=0.968$ ,  $p<0.001$ ,  $n=9$ ), but not TAX1BP1 expression (Fig. 2E).

#### 4. Discussion

A20 is an important regulator of inflammatory signalling, which inhibits p65 translocation to the nucleus. Conversely, silencing of A20 increases p65 activation and potentiates the inflammatory response [12]. The ability of A20 to inhibit NF- $\kappa$ B activation is dependent on interaction with the TRAF6 binding protein TAX1BP1 [13,14]. In complex, A20 and TAX1BP1 act directly to prevent TRAF6 polyubiquitination and terminate TLR-driven inflammatory signalling [14]. In the absence of TAX1BP1, A20 cannot bind or act on target proteins [13], leading to persistent NF- $\kappa$ B activation and chronic inflammation. Using LPS stimulated CFBE41o cells we have previously shown that A20 does not interact with TAX1BP1 or TRAF6. This, together with persistently upregulated nuclear p65 protein expression, suggests that A20 action is dysfunctional in CF epithelial cells [1].

Here, we extend our previous findings to different CF genotypes and investigate the association of reduced A20/TAX1BP1 expression with levels of inflammation and lung function (FEV<sub>1</sub>). Basally and following LPS stimulation, A20 expression was reduced in CF cells in a manner that largely reflected genotype; the F508del/F508del group had a lower expression than the R117H/F508del group. Furthermore, basal A20 expression in CF patients (both genotypes) was inversely proportional to p65 levels. LPS induces A20, which in turn inhibits NF- $\kappa$ B activity. LPS selectively increases the transcriptional activity of p65, but not p50, in intestinal epithelial cells [15]. In line with this, overexpression of p65 increases NF- $\kappa$ B transcriptional activity [16]. Furthermore, A20 is itself regulated by NF- $\kappa$ B and p65 has been shown to bind to endogenous A20 [17]. Our previous work has shown increased and sustained activation of p65 in CF airway epithelial cells [1]. We therefore measured p65 mRNA expression to provide an indication of how reduced A20 expression may relate to NF- $\kappa$ B and the inflammatory response. Our results suggest that A20 expression may be directly related to CFTR expression/function and underlines the important role for A20 in the regulation of NF- $\kappa$ B activation in CF.

Finally, and in line with the role of A20 in regulating inflammatory responses, our data show a significant correlation between FEV<sub>1</sub> and basal A20 expression. Notably, those with the poorest lung function were those with the most pronounced reduction in A20 expression irrespective of genotype. Data from CFTR silenced 16HBE14o- cells support a possible direct link between A20 and CFTR. Our work suggests that CFTR expression can modify the transcription of A20; however, the mechanism of this modification is not yet known. We are examining a number of potential intermediaries including the A20 binding inhibitors of NF- $\kappa$ B (ABIN) proteins. Although TAX1BP1 expression was not altered by CFTR silencing, the genotype specific expression profile would suggest that TAX1BP1 is regulated by an intermediary (potentially TRAF6 or other protein(s) within the TRAF6 feedback loop), which will also be investigated in future.

In conclusion, LPS-stimulated expression of A20 mRNA is reduced in CF in a manner that broadly reflects genotype. More specifically, basal A20 expression is altered in a manner proportional to FEV<sub>1</sub>. Pending confirmation in a larger scale study, A20 may prove a novel predictor of inflammation and disease severity in the CF population and future studies will investigate if pharmacological induction of A20 can modulate NF- $\kappa$ B driven inflammation in CF airways.

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