THE C.1766+1G→A SPLICE SITE MUTATION CAUSES EXON 13 SKIPPING RESULTING IN MUTIPLE DEFECTS IN CFTR STRUCTURE AND FUNCTION

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Abstract

Splice site mutations account for 12% of the over 2000 CFTR gene mutations identified in cystic fibrosis (CF) patients. The C.1766+1G→A splice site mutation is one of the 16 most common CFTR mutations worldwide. In the CFTR2 global database, ~0.6% of patients with CF have at least one C.1766+1G→A allele. However, the structural and functional defects of CFTR caused by this splice site mutation at the donor site of intron 13 remains unclear. In addition, the effects of CFTR correctors on this splice site mutant remain to be elucidated. To characterize C.1766+1G→A CFTR and explore potential therapeutic strategies, we studied heterologous primary hBE cells with the F508del and C.1766+1G→A CFTR mutations (patient code: CFBr002). RT-PCR analysis indicated that this splice site mutation causes CFTR exon13 skipping (87bp), which results in a deletion of 29 amino acids from Arg560 to Ser589 in CFTR. This missing segment is located at NBD1 domain and contains the Walker B motif in the ATP binding site 1. These results are consistent with early reports by Strong, T.V. et al. (Human Mutation, 1(5):380-7 (1992)). Moreover, F508del and C.1766+1G→A CFTR genes in this heterozygous donor encoded Met470 and Val470, respectively. Equivalent TECC current studies indicated there were no effects of forskolin and potentiators on these heterozygous primary hBE cells in the absence of CFTR correctors. Compared to homozygous F508del-CFTR primary hBE (patient code: CFFT028H), the combination of CFTR correctors and potentiators have lower efficacy in CFBr002, suggesting that C.1766+1G→A CFTR has impaired chloride channel function.

To further investigate this splice site mutant, C.1766+1G→A CFTR was cloned into an expression vector, pcDNA3.1(+), and transfected into immortalized hBE (CFBE), FRT and HEK293 cells, and stable cell lines expressing the C.1766+1G→A were established. Western blot analyses revealed only a weak Band B in the three C.1766+1G→A stable cell lines compared to WT-CFTR expressing cells, suggesting the splice site mutant causes impaired biogenesis and maturation defects. Common F508del CFTR correctors (C7 (PTC124), C17, C18) and a series of AbbVie compounds (such as AC1, AC2a, AC2b, and AC2c) significantly increased C.1766+1G→A Band B, but did not produce Band C individually. However, a combination of two compounds, such as AC1/AC2c and C18/AC2c, resulted in the production of Band C in CFBEs, suggesting that these compounds work together to promote C.1766+1G→A CFTR maturation. The rescued band C protein could not be activated by forskolin or forskolin plus several different CFTR potentiators, including VX-770, PG-01, and Genistein, implying C.1766+1G→A channel function is also defective. In conclusion, the C.1766+1G→A CFTR splice site mutation results in multiple defects including protein biogenesis, maturation and channel function.

Methods

Cell Culture **:** CF patient primary hBE, immortalized CFBE, FRT , and HEK293 cells were grown in BEGM, EMEM, Ham's F-12, and DMEM, respectively, at 37°C and 5% CO_2. Vertex differentiation media is used for primary hBE air liquid interface culture.

Molecular Biology and Biochemical Assay: Total RNA extraction from primary hBE, PCR, and RT-PCR analyses were performed by QIAGEN and ThermoScrip RT-PCR system. CFBE, FRT, and HEK293 cells were stably transfected with pcDNA3.1(+)/neo-C.1766+1G→A CFTR using Lipofectamine 2000. CFTR Expression in various cells was imaged by confocal microcopy, LSM 880(ZEISS). Lysis buffer (0.4% sodium deoxycholate, 1%NP-40, 50mM Tris-HCl, pH 7.4) with protease inhibitors. Cell lysates were subjected to Western blotting. CFTR antibody, 596, was used for a series of biochemical detections, such as imaging and Western blotting.

Equivalent Current (I_{eq}) and Conductance (G_t) Measurements: Cells were seeded onto HTS Transwell®-24 well permeable filter plates (0.4 uM pore size, Polyester,Corning). I_{eq} measurements were carried out 35-45 days after seeding. G_t assays for CFBE and FRT were performed between 7 and 10 days after seeding, respectively. The data were recorded with 24-channel transepithelial current clamp (TECC)_Robot system (Design, Belgium). Assay buffer is Ham's F-12 without FBS and $NaHCO_3$. Student's *t* test was used to determine the statistical significance of the quantitative results. Results with a *P* value < 0.05 were considered statistically significant.

Results

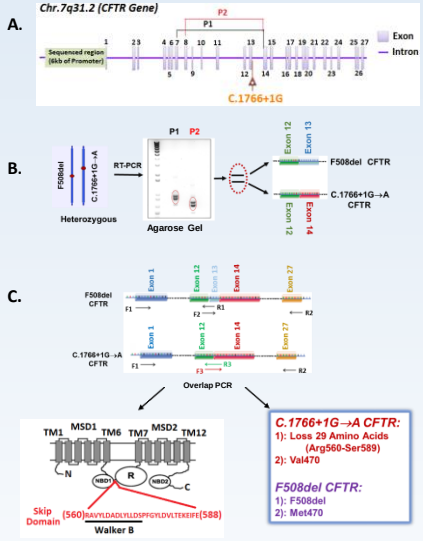

Figure 1 Identification of Heterozygous C.1766+1G→A/F508del CFTR in Cystic Fibrosis Primary hBE(Patient Code: CFBr002).

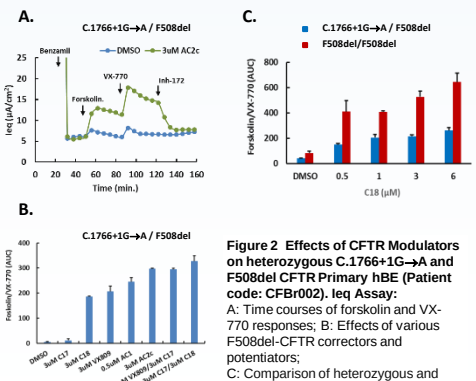

Figure 2 Effects of CFTR Modulators on heterozygous C.1766+1G→A and F508del CFTR Primary hBE (Patient code: CFBr002). Ieq Assay: A: Time courses of forskolin and VX-770 responses; B: Effects of various F508del-CFTR correctors and potentiators;
C: Comparison of heterozygous and homozygous(F508del) CFTR hBEs.

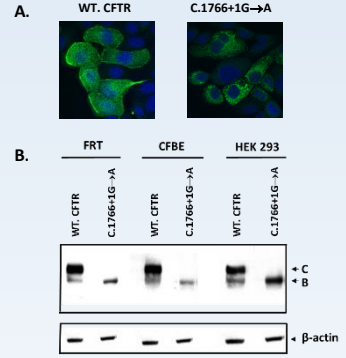

Figure 3 Expression of C.1766+1G→A CFTR Mutant in Various Cell Lines. A. Confocal imaging in FRT cells ; **B.** Western Blotting.

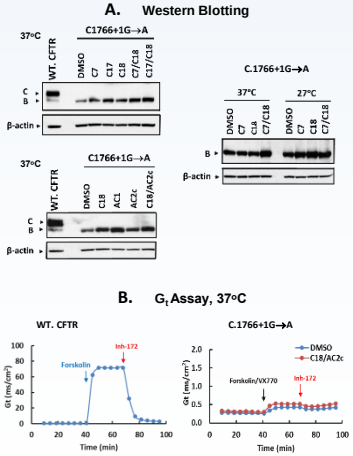

Figure 4 Effects of CFTR Modulators on C.1766+1G→A CFTR Mutant in FRT Cells. CFTR modulator concentration: 10μM C7, 6μM C17, 6μM C18, 1 μM AC1, 3 μM AC2c, 10 μM Forskolin, 1 μM VX-770, and 10 μM CFTR inh-172.

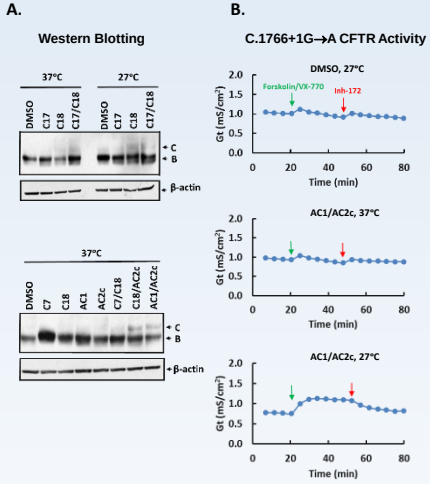

Figure 5 Effects of CFTR Modulators on C.1766+1G→A CFTR Mutant in Immortalized CFBE Cells. CFTR modulator concentration: 10μM C7, 6μM C17, 6μM C18, 1 μM AC1, 3 μM AC2c, 10 μM Forskolin, 1 μM VX-770, and 10 μM CFTR inh.172.

Conclusions

1. **C.1766+1G→A splice site mutation causes CFTR exon 13 skipping and the loss of 29 amino acids from Arg560-Ser589 in NBD1 .**
2. **Exon 13 skipping results in multiple defects including biogenesis, folding, trafficking, and channel function.**
3. **Although combinations of CFTR correctors can rescue misfolding and trafficking of C.1766+1G→A CFTR, available CFTR potentiators cannot repair its defective channel function at 37°C.**

References

1. **Strong, T.V. et al. Human Mutation, 1992, 1(5):380-387.**
2. **Hull, J. et al. Human Mol. Genet. 1993, 2(6): 689-692.**