CFTR transcripts are undetectable in lymphocytes and respiratory epithelial cells of a CF patient homozygous for the nonsense mutation R553X

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Abstract

In order to analyse the influence of the nonsense mutation R553X on CFTR gene expression, transcripts from epithelial cells and lymphocytes were examined from nine subjects (one CF patient homozygous for R553X, one CF patient compound heterozygous for R553X/ΔF508, four CF carriers heterozygous for R553X, one CF carrier with the genotype ΔF508/N, and two uncharacterised normal adults). After reverse transcription of the region from exons 10 to 13 to cDNA, fragments of the expected size were amplified from all heterozygous and normal subjects. In three subjects an additional alternatively spliced product was observed, which was found to contain a termination codon. In repeated experiments it was not possible to detect any CFTR mRNA in cells derived from the R553X homozygous patient. Furthermore, in subjects heterozygous for R553X we could not detect by hybridisation with a specific oligonucleotide probe and direct sequencing any CFTR mRNA derived from the R553X allele. However, the wild type product was present in all of these subjects. Our results support the view that nonsense mutations in the CFTR gene can lead to a reduction or absence of cytoplasmic CFTR mRNA.

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Cystic fibrosis (CF) is one of the most common autosomal recessive disorders in white populations. In CF patients, abnormal chloride secretion across the apical membrane of the epithelial cells causes clinical symptoms, including chronic pulmonary disease and pancreatic exocrine insufficiency, together with an increase in the concentration of sweat electrolytes.[1] The gene responsible for CF, the cystic fibrosis transmembrane conductance regulator (CFTR) gene, is located on chromosome 7q31 and has been cloned and sequenced.[2,3] The CFTR protein shows structural similarities to the members of the ATP binding transporter family. The function of CFTR is not completely understood, but the results of experiments involving expression of normal CFTR in human, rodent, and insect cells suggest that CFTR itself is a chloride channel.[4,5] Over 200 different mutations in the CFTR gene associated with the CF phenotype have been reported so far (Cystic Fibrosis Genetics Analysis Consortium, June 1992). The most frequent mutation, ΔF508,[6] located in the first nucleotide binding fold, is usually associated with severe CF,[7] but in exceptional cases ΔF508 homozygotes are only mildly affected.[8]

Three nonsense mutations, G542X,[9] R553X,[10] and W1282X,[11] represent the second, fourth, and fifth most frequent CF mutations worldwide (Cystic Fibrosis Genetics Analysis Consortium, unpublished data). Until now, only a few cases of homozygosity for nonsense mutations or compound heterozygosity for two nonsense mutations have been reported. Two CF patients, homozygous for the G542X mutation, have been described as mildly affected.[12,13] Cutting *et al*[14] found two patients heterozygous for W1316X/R553X and S1255X/G542X, respectively, to exhibit severe pancreatic but only mild pulmonary disease. In contrast, Shoshani *et al*[15] showed that the most common CF mutation in the Ashkenazi Jewish population, W1282X, which is located in exon 20, is clearly associated with a severe phenotype. We previously classified the only known homozygous R553X patient as being moderately severely affected.[16] We report here on CFTR mRNA analysis in this patient, who is homozygous for R553X (a CGA to TGA substitution at position 1789 in exon 11 of the CFTR gene), and in R553X heterozygotes, in order to investigate the influence of nonsense mutations on gene expression.

Methods

FAMILY A

Case 1 was a 15 year old male CF patient of German origin, who was homozygous for the stop mutation R553X, with severely reduced pancreatic function, but only mildly affected lung function. His height was normal, but his weight ranged between the 3rd and the 10th centile. We have previously described the clinical features of this patient in greater detail.[16] Case 2 was a male CF carrier (R553X) of German origin. He was a symptomless adult and the father of case 1 and a second cousin of case 3. Case 3 was a female CF carrier (R553X) of German origin. She was a symptomless adult and the mother of case 1 and a second cousin of case 2.

FAMILY B

Case 4 was a 6 year old male CF patient of German origin and a compound heterozygote for ΔF508 and R553X, with insufficient pancreatic function and recurrent pneumonia. His weight is below the 3rd centile, while his height is normal. We classify this patient as being severely affected. Case 5 was a male CF carrier (ΔF508) of German origin. He was a symptomless adult and the father of case 4. Case 6 was a female CF carrier (R553X) of German origin. She was a symptomless adult and the mother of case 4.

CONTROLS

Case 7 was a male CF carrier (R553X) of American origin and was a symptomless adult. Case 8 was a female of German origin and was a symptomless adult. Case 9 was a male of Polish origin and was a symptomless adult.

From cases 1, 3, 4, 5, 6, 8, and 9 nasal epithelial cells were obtained by brushing and blood was also collected from these persons. Lymphocytes were obtained from case 2 (from fresh blood) and case 7 (from a lymphoblastoid cell culture).

RNA EXTRACTION AND cDNA SYNTHESIS

Cells obtained by brushing were either transferred to culture medium, centrifuged, washed with PBS, and resuspended in 4 mol/l guanidinium thiocyanate buffer (GTC), or were placed directly into 4 mol/l GTC. Total RNA was extracted from pelleted lymphocytes and epithelial cells using the guanidinium thioisocyanate method.[17] To control the mRNA quality, aliquots of all samples were reverse transcribed to cDNA using a primer within (1) the multidrug resistance (MDR1) gene and (2) the β2-microglobulin (β2m) gene and afterwards amplified with primers located in appropriate regions of these genes.[18] Only mRNA preparations from which fragments of the expected size could be amplified were used in the following experiments. A control transcription of a sample without RNA was included in each cDNA synthesis.

For reverse transcription of CFTR mRNA primer 13c (the sequences of the oligonucleotides used in this study are listed in the table) was used. Total RNA (5 μg) or mRNA (0·5 μg) were specifically converted to cDNA. We followed the reverse transcription protocol suggested for the GeneAmpRNA PCR Kit (Perkin Elmer Cetus) and incubated 20 μl cDNA reaction mixture (including 2 U Ampli*Taq* DNA polymerase) in a Perkin-Elmer Cetus DNA Thermal Cycler at 42°C for 15 minutes, 99°C for five minutes, and 5°C for five minutes.

DNA sequence of the oligonucleotides used for reverse transcription (RT), polymerase chain reaction (PCR), and allele specific oligonucleotide (ASO) hybridisation.

Oligonucleotide	Sequence	
13c	5-GTCGAAAGAATCACATCCCATGA-3	(RT, PCR)
13MS	5-TCACATCCCATGAGTTTTGAGCT-3	(PCR)
8a	5-GTAGTGATGGAGAATGTAACAGCC-3	(PCR)
9MF	5-TGTAAAACGACGGCCAGTAATGGTGATGACAGC-3	(PCR)
10c	5-GTTTTCCTGGATTATGCCTGGCACC	(PCR)
R553X mutant	5-GGAGGTCAATGAGCA-3	(ASO)
R553X wild type	5-GGAGGTCAACGAGCA-3	(ASO)

'NESTED' POLYMERASE CHAIN REACTION (PCR)

An initial PCR was performed with 20 μl cDNA, primers 8a and 13c (30 pmol each), 5 μl 10 × *Taq* buffer, 2·5 U *Taq* polymerase (Stratagene), and 0·5 μl 20 mmol/l (each) dNTP (Boehringer) in a total volume of 50 μl under the following conditions: 10 minutes' denaturation at 94°C, 35 cycles consisting of one minute denaturation at 94°C, one minute annealing at 55°C, and three minutes' extension at 72°C. Final extension was at 72°C for 10 minutes. Under identical conditions, two nested amplifications were performed on a fixed aliquot (5%) of the total PCR volume with the primer combinations 13MS/9MF and 13MS/10c. Ten percent of the total PCR reaction was electrophoresed through a 0·8% agarose gel stained with ethidium bromide.

SEQUENCING OF THE AMPLIFIED cDNA

The PCR products were size fractionated on an 8% polyacrylamide gel in 1 × TBE buffer. The bands were cut out and eluted overnight in distilled water at 60°C. Under the conditions described above, the cDNA was subjected to an asymmetrical PCR reaction (primers 13MS/10c with a ratio of 100:1). After centrifugation of the PCR products through Centricon-100 columns, direct sequencing with Sequenase (USB) was carried out according to the manufacturer's instructions.

ALLELE SPECIFIC OLIGONUCLEOTIDE (ASO) HYBRIDISATION

The amplified cDNA from all probands was dot blotted to nylon membranes (Hybond N+, Amersham) according to standard procedures.[19] Filters were prehybridised for one hour at 60°C (ASO for wild type allele) and 62°C (R553X mutation specific ASO) in a hybridisation solution containing 5 × SSPE, 5 × Denhardt's solution, and 0·5% Triton X-100. The labelling reaction of the oligonucleotides was performed at 37°C for 30 minutes. The labelling mixture included 10 μl oligonucleotide (1 0D/ml), 3 μl 10 × kinase buffer, 15 μl ^{32}P dATP, and 2 μl T4 polynucleotide kinase (Boehringer). After prehybridisation, the filters were hybridised at the same temperature for one hour. The filters were washed once at room temperature with a 2 × SSPE, 0·1% Triton X-100 solution for two minutes followed by a final wash with the same solution for 15 minutes at 60°C and 62°C, respectively. Subsequently the filters were exposed to *x* ray film for one hour. As positive controls, PCR products including exon 11, amplified with primers 11i-5 and 11i-3[20] from genomic DNA of a R553X carrier, a patient homozygous for R553X, and a normal adult were blotted and hybridised.

Results

We converted CFTR mRNA from lymphocytes and nasal epithelial cells to cDNA and amplified the region from exons 10 to 13 by PCR. Since the amount of CFTR transcripts in 'non-expressing' tissues such as lymphocytes is very low, we used the 'nested' PCR approach (fig 1). With the exception of the R553X homozygous patient (lane 5), cDNA amplification was possible from all nasal epithelial cells and lymphocytes that were attempted. Fragments of three different lengths, measuring 583, 464, and 377 bp, were seen (fig 2). All PCR products were analysed by direct sequencing. The 464 bp fragment was identified as the expected normal splice product. The 583 bp product was found to be the result of alternative splicing and included a 119 bp insert with a stop codon. We have previously shown[21] that the occurrence of the splice variant appears to be independent of the CFTR genotype. In the 377 bp fragment, exon 12 is missing. Exon 12 skipping, accounting for over 90% of the detectable mRNA, has been previously reported.[22,23] In repeated experiments, it was not possible to detect any CFTR cDNA from the R553X homozygous patient on ethidium bromide stained agarose gel. This indicates a severe reduction or even absence of the CFTR mRNA in the homozygous patient. Control RT-PCR experiments using primers from within the multidrug resistance gene (MDR1) and the β2-microglobulin gene (β2m) showed that the mRNA from this patient was intact. In order to increase the level of detection sensitivity, we performed allele specific oligonucleotide (ASO) hybridisations for the R553X and normal alleles. As shown in fig 3, only the CFTR exon 11 controls, amplified from genomic DNA of homozygotes and heterozygotes for R553X, hybridised to the R553X mutant oligonucleotide. CFTR exon 11 DNA of a person heterozygous for R553X hybridised to both the wild type and the mutant oligonucleotides. The wild type ASO hybridised to the amplified cDNA from the region of exons 10 to 13 from the normal and heterozygous subjects. The PCR reaction from the homozygous patient hybridised to neither the normal nor the R553X ASO. Furthermore, by direct sequencing of the PCR products of heterozygotes from mRNA of both nasal epithelial cells and lymphocytes only the wild type sequence was obtained. These results show that the levels of CFTR transcripts carrying the R553X mutation are severely reduced or even absent in both tissues.

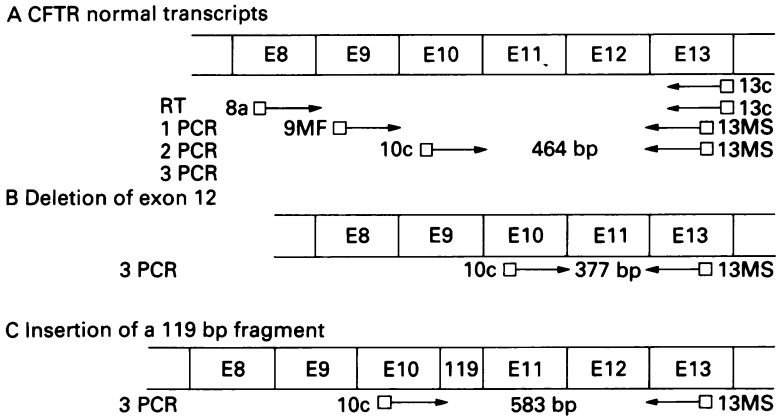

Figure 1 Schematic representation of the 'nested' PCR approach. Total RNA was converted to cDNA using primer 13c and amplified by 'nested' PCR with the primer sets 8a/13c, 9MF/13MS, and 10c/13MS. (A) CFTR PCR products of the expected size (464 bp) include parts of exon 10, exon 11, exon 12, and exon 13. (B) In the 377 bp PCR product, exon 12 is deleted. (C) The 583 bp alternative spliced fragment contains a 119 bp insertion directly between exon 10 and exon 11.

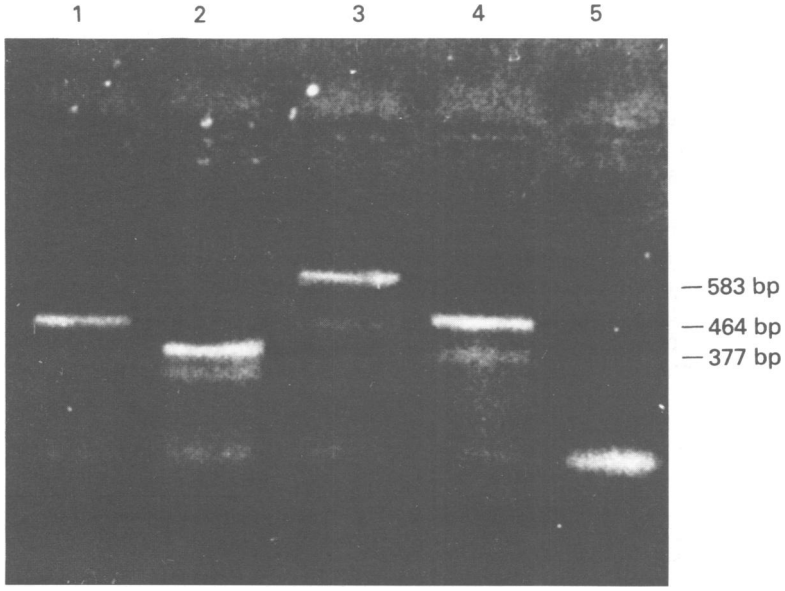

Figure 2 Alternative splicing in the first nucleotide binding fold of CFTR. (A) 0·8% agarose gel of 'nested' PCR products amplified from the R553X homozygous patient (lane 5), the patient's mother (lane 3), the patient's father (lane 4), and two healthy controls (lanes 1 and 2). Products of 583 bp, 464 bp, and 377 bp in size are visible. The 583 bp product included a 119 bp insert between exons 10 and 11. The 464 bp fragment represents the expected, normally spliced product. In the 377 bp fragment, exon 12 is missing. In lane 5, the primers are present (bottom), but no amplification product is detectable.

Discussion

We show here that patients, either homozygous or heterozygous for the nonsense mutation R553X, have no detectable CFTR mRNA derived from the R553X allele in nasal epithelial cells and lymphocytes. This observation is in accordance with previous findings of Hamosh *et al*[24] and Jones *et al*[25] for other nonsense mutations within the CFTR gene. Furthermore, a number of nonsense mutations in other eukaryotic genes (CYP21B gene, β globin gene, insulin receptor gene) have been shown to be associated with a severely reduced and thereby undetectable mRNA level.[26-28] The mechanisms by which nonsense mutations reduce the cellular mRNA levels are not well understood. A lower transcription rate, a lower rate of RNA processing including a rapid destruction of unstable intermediates, a reduced transport from spliced mRNA, or a decreased stability of mature mRNA have all been discussed.

Sequence changes have been shown to affect the stability of mRNA in bacteria and yeast.[29] However, Urlaub *et al*[30] have ruled out an increased lability of dihydrofolate reductase mRNA in eukaryotic cells carrying stop codon mutations. Moreover, these authors observed the 'polar' effect that nonsense mutations within the first exons of this gene lead to

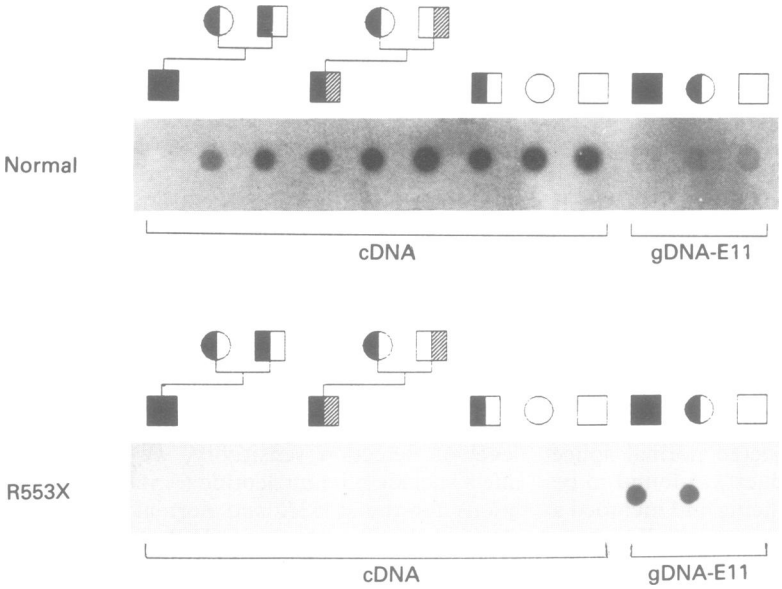

Figure 3 Allele specific oligonucleotide hybridisation (ASO) for the R553X and normal alleles. Each membrane was dotted with amplified cDNAs from all subjects (subjects 1 to 9 from left to right) and PCR products including exon 11, amplified with primers 11i-5 and 11i-3[20] from genomic DNA of the R553X homozygous patient, a R553X carrier, and a normal adult. Hybridisation was carried out with an ASO for the wild type allele (normal) and a R553X mutation specific ASO (R553X). Solid symbols: R553X, hatched symbols: ΔF508, open symbols: normal.

drastically reduced intracytoplasmic levels of dihydrofolate reductase mRNA, whereas stop codon mutations in the last exon result in normal mRNA levels. It was possible to restore normal RNA levels in revertants of low RNA nonsense mutants by substitution of the nonsense mutants through missense mutants.[30] This finding suggests that the low mRNA level is a functional consequence of the nonsense codons and not caused by a change in mRNA secondary structure. If stop codon mutations, such as R553X, within the first half of the CFTR gene, generally lead to severely reduced or even absent intracytoplasmic mRNA levels, it is surprising that the 583 bp splice variant carrying a stop codon within the same region appears to be rather stable[21] (this study). Our data suggest the existence of special recognition signals of the post-transcriptional process (for example, for transport from the nucleus into the cytoplasm), which are not affected by the stop codon of the splicing variant.

In summary, we assume that an association exists between the intracytoplasmic mRNA level and the phenotype. The patients seem to be less severely affected if, because of homozygosity or compound heterozygosity for nonsense mutations, the intracytoplasmic mRNA is reduced or absent. However, this view may be biased because of the small number of patients studied in this respect. Transcription studies of patients homozygous for the stop mutation W1282X within exon 20 of the CFTR gene (that is, its 3′ end), which is associated with a severe phenotype,[15] as well as mRNA analysis of other patients homozygous for nonsense mutations will help to come to a better understanding of the relationship between the location of nonsense mutations, and their influence on the mRNA level and the clinical phenotype.

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