ORIGINAL ARTICLE

Detection of a large heterozygous deletion and a splicing defect in the CFTR transcripts from nasal swab of a Japanese case of cystic fibrosis

Miyuki Nakakuki¹, Kotoyo Fujiki², Akiko Yamamoto¹, Shigeru BH Ko³, Lanjuan Yi¹, Mariko Ishiguro¹, Makoto Yamaguchi¹, Shiho Kondo¹, Shinsuke Maruyama⁴, Kosuke Yanagimoto⁴, Satoru Naruse⁵ and Hiroshi Ishiguro¹

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in CFTR (CF transmembrane conductance regulator). Although CF is the most common hereditary disease in Caucasians, it is rare in Asian populations. Common disease-causing mutations of CFTR in Caucasians are rarely identified in Japanese patients with CF. In the present study, CFTR transcripts from nasal swab were analyzed in a Japanese boy, in addition to conventional PCR and direct sequence of all exons, their boundaries and promoter region of the CFTR gene. The boy was diagnosed with CF by chronic respiratory infection and the elevated sweat chloride level. None of the disease-causing mutations of CFTR was detected by the conventional analysis. Cloning and sequence of the CFTR transcripts revealed a heterozygous deletion spanning exons 16, 17a and 17b. The deletion was confirmed by multiplex ligation-dependent probe amplification and the direct sequence of the junction fragment obtained from the genomic DNA by primer walking, which revealed the mutation c.2908 + 1085_3367 + 260del7201. We also identified a splicing defect: deletion/skipping of exon 1 in the CFTR transcript from the other allele. The analysis of CFTR transcripts from nasal swab is recommended in the genetic analysis of CF in Japanese.

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INTRODUCTION

Cystic fibrosis (CF; MIM no. 219700) is an autosomal recessive disease caused by mutations in CF transmembrane conductance regulator (CFTR). CFTR is expressed in the apical membrane of various epithelia and functions as a cyclic AMP-regulated anion channel. Loss-of-function due to severe mutations in both alleles causes dehydrated, thick and viscous luminal fluid in respiratory and gastrointestinal tract, pancreatic duct and vas deferens. CF typically involves chronic infection and progressive obstruction of the respiratory tract, pancreatic exocrine insufficiency and male infertility. CFTR mediates Cl[−] absorption by sweat duct, and the elevated sweat chloride is the gold standard for CF diagnosis.

CF is one of the most common hereditary diseases in Caucasians with an estimated incidence of 1 in 2500–3500 newborns. Diagnosis of CF can be made if the sweat chloride value is ≥ 60 mmol l^{−1} in individuals presenting one or more characteristic symptoms or a positive family history.¹ Individuals with sweat chloride values in the

intermediate range should undergo analysis of CFTR mutations.² The American College of Medical Genetics recommends a screening panel of 23 types of CF-causing mutations, which detects 85–88% of CF alleles in Caucasians.^{3,4} However, there are considerable regional and ethnic variations in the spectrum of the CFTR mutations.⁵

CF is very rare in Japan approximately three per million.^{6,7} Definite diagnosis of CF is often difficult for Japanese patients because (1) sweat Cl[−] test with pilocarpine iontophoresis is not readily available in Japan and (2) most of the CFTR mutations are of rare types. The whole coding regions (27 exons) of the CFTR gene are scanned by single-strand conformational polymorphism analysis and/or direct sequence, when CF is suspected in Japanese.⁸ However, a recent national surveillance of CF in Japan revealed that the presence of mutations in a substantial number of highly suspected cases remained unsolved (Research Committee of Intractable Pancreatic Diseases, data not published).

More than 1800 different mutations have been reported to the Cystic Fibrosis Mutation Database (www.genet.sickkids.on.ca/cftr),

¹Department of Human Nutrition, Nagoya University Graduate School of Medicine, Research Center of Health, Physical Fitness and Sports, Nagoya University, Nagoya, Japan; ²Department of Nutritional Sciences, Nagoya University of Arts and Sciences, Nisshin, Japan; ³Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan; ⁴Department of Pediatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan and ⁵Miyoshi Municipal Hospital, Miyoshi, Japan

Correspondence: Dr H Ishiguro, Department of Human Nutrition, Nagoya University Graduate School of Medicine, Research Center of Health, Physical Fitness and Sports, Nagoya University, Furo-cho E5-2 (130), Chikusa-ku, Nagoya 464-8601, Japan.

E-mail: ishiguro@htc.nagoya-u.ac.jp

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which includes 39 different genomic rearrangements, such as large deletion. Intragenic rearrangements (large deletions and to lesser extent, insertions) account for 1–3% of the CFTR mutations in Caucasians.⁴ Large deletions in heterozygosis escape detection by conventional PCR-based techniques. In the present study, we detected a large heterozygous deletion spanning exons 16, 17a and 17b in a Japanese CF patient by analysis of CFTR transcripts extracted from nasal swab. The deletion was confirmed by the direct sequence of the junction fragment obtained by primer walking, which revealed the mutation c.2908 + 1085_3367 + 260del7201. The deletion was previously reported from a French CF patient and his healthy mother, who came from South Korea.⁹

Analysis of the CFTR transcripts is also useful to detect splicing defects and reduced mRNA expression. More than 15% of disease-associated CFTR mutations modulate pre-mRNA splicing.¹⁰ In the present study, we have identified a novel splicing defect in the CFTR transcript from the allele not lacking exons 16–17b. The deletion/skipping of exon 1 by a splicing defect has not been reported in CF patients.

The screening of large genomic deletion and splicing defect by transcript analysis of nasal swab is recommended in the genetic analysis of CF in Japan and Asian countries, where common CF-causing mutations in Caucasians are rarely identified.

MATERIALS AND METHODS

The following study was approved by the Ethical Committee of Nagoya University Graduate School of Medicine.

Case report

A 1-year-old boy was admitted to hospital for the treatment of poor nutritional status. He is the first boy child of non-consanguineous Japanese parents. He had an operation for meconium ileus on the first day after birth, intrahepatic cholestasis and recurrent pneumonia. Mucoid strains of *Pseudomonas aeruginosa* were isolated from the sputa. Sudan stain of fecal fat was positive. Sweat Cl^- concentration was elevated (122 mmol l^{-1}) and he was diagnosed with CF.

Direct sequence of the CFTR gene

Genomic DNA was extracted from peripheral leukocytes. PCR was carried out using the primers shown in Table 1. All 27 exons and their boundaries (100–300 bp including poly T and TG repeats in intron 8) and promoter region (up to 1028 bp upstream of the translation initiation codon of exon 1) were directly sequenced.

Cloning of the CFTR transcripts from nasal swab

Nasal epithelial cells were collected by gently brushing the inferior turbinates of both nostrils using a cotton swab.¹¹ The swab was immediately immersed in the Buffer RLT preservation solution (Qiagen, Hilden, Germany) and stored at -20°C . Total RNA was extracted (750–3500 ng from one sample) and reverse transcribed to cDNA using two kinds of reverse transcriptase-primer; a random 6mer and the gene-specific primer on exon 24 (5'-TCTGGCTTGCAAAACACAAG-3'). The cDNA that was generated using the random 6mer was amplified by PCR into 11 overlapping segments. Positions, primer sequences and predicted sizes of the segments are shown in Figure 1a and Table 2. The cDNA generated using the gene-specific primer on exon 24 was subjected to long PCR analysis. Eight sets of long PCR (Figures 4a and e) were performed using the primers described in Table 3. The PCR products were subject to agarose gel electrophoresis. The bands of predicted sizes were excised and purified.

The purified PCR products were cloned using the Mighty TA-cloning Kit (Takara Bio, Otsu, Japan). Colonies were screened for correct insertion of the plasmid by PCR and cultured for 14–16 h. The plasmid was extracted with the GeneElute Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The

Table 1 Primer sequences for conventional PCR analysis of the CFTR gene

Exon	Forward primer (5'–3')	Reverse primer (5'–3')
5'UTR	GCCCCTCAGAGAGTTGAAGA	TGATCCTAGTCGGGTTCCCTG
5'UTR	AAACGTAACAGGAACCCGACTA	CTCAACCCCTTTTCTCTGACCT
Exon 1	GGAGAAAGCCGCTAGAGCAAA	GTGGCTCTCTATTCAATCAGC
Exon 2	TATTCCTCCCAATCCCTTT	TGGGATTACAGGCATTAGCC
Exon 2	TGTAAGAGATGAAGCCTGGTA	GCTCCTATTTTAAATATAAG
Exon 3	CCATGAGATTTTGTCTCTATA	GAGTTGGATTCACTCTTTATA
Exon 4	AAGAGTTTCACATATGGTATG	TGCCATTATTTAATAGGCAT
Exon 5	GAAGATAGTAAGCTAGATGAA	AATTGACCTTTCTTAGTTTCC
Exon 6a	TGCTCAGAACCACGAAGTGT	ATTAGCTGGGTGTGGTGCAT
		CTGAGGCAGGAGAATTGCTT
Exon 6b	TGGAATGAGTCTGTACAGCG	TGCATGAATATTGACAGAACT
Exon 7	TATAGGCAGAAAGACTCTAGA	TCCTAGTATTAGCTGGCAACT
Exon 8	TGGGTAATTACAGGGTTGCTT	CGCCATTAGGATGAAATCCA
Exon 9	GGCCATGTGCTTTTCAAACCT	TCGCCATGTGCAAGATACAG
Exon 10	TTGTGCATAGCAGAGTACCTGAAA	ATTGATCCATTACAGTAGCT
Exon 11	TCAGCAATGTTGTTTTGACC	CCAAGATACGGGCACAGATT
Exon 12	CTTCTGCACCACTTTTGAGAA	GCTACATTCTGCCATACCAA
Exon 13	GGTACCAATTAATACTACAG	ATTTGTAAGGGAGTCTTTTGC
Exon 13	TGGGATGTGATTCTTTCCACC	GGGAAGAGATATGCTCATTCG
Exon 13	CATTAGAAGGAGATGCTCCTGT	TCGTAATCCTATGATTTAGT
Exon 14a	ACACTTAGATTCAAGTAATACT	CAGAAGCTAAGAACTATATGA
Exon 14b	GACCCAGGAACAAAGCAAA	TTCCACTACCATAATGCTTGG
Exon 15	GGTTAAGGGTGCTGCTCTTC	AAGCCAGCACTGCCATTA
Exon 15	TAGACTCAAGTTTAGTTCCATT	TTAGAAAACCAACAAAACCA
Exon 16	TCTGAATGCGTCTACTGTGA	GCAATAGACAGGACTTCAAC
Exon 17a	CACCAGCATGGCACATGTAT	CCAAAATGAAGTCACATGGTCA
Exon 17a	AGAAATAAATCACTGACACAC	CCATGTGTACTTTGTAATATAG
Exon 17b	CAAGAATGGCACCAGTGTG	CGACAATCTGTGTGCATCG
Exon 17b	TTTAACCAATGACATTGTGA	ATACCGATTCAAGGAAATTA
Exon 18	TAGGAGAAGTGTGAATAAAG	GATACACAGTGACCCTCAATT
Exon 19	GCCCGACAAATAACCAAGTG	TGCTTCAGGCTACTGGGATT
Exon 20	CAGGATTGAAAGTGTGCAACA	CTATGAGAAAACGCACTGGA
Exon 21	TGTTCAACAAGGACTCCAAA	AGGGGTAGGTCAGTCAAAA
Exon 22	AGAGCCATGTGCCACGTATT	TCCATGGGCAATTATTTCAT
Exon 23	CCATGGTTGAAAAGCTGATTG	TGAGTAAAGCTGGATGGCTGT
Exon 24	TTTCTGTCCCTGCTCTGGTC	TCTGGCTTGCAAAACACAAG
		ACTATTGCCAGGAAGCCATT

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; UTR, untranslated region.

sequence reaction was carried out using the GenomeLab Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter, Fullerton, CA, USA). The reaction products were purified using the Centri-Sep spin columns (Applied Biosystems, Grand Island, NY, USA). Capillary electrophoresis and sequence was performed by CEQ 8000 system (Beckman Coulter).

Analysis of the genomic rearrangement of CFTR

Long PCR from exon 15 to exon 18 of the CFTR gene was performed using the forward primer on exon 15: 5'-CCAGCACCAGTTCGTATTATGTGTTTT-3' and the reverse primer on exon 18: 5'-AGCCCACTGCAATGTACTCATGATATT-3'. The PCR protocol was polymerase activation at 94°C for 5 min, followed by 35 cycles of PCR with denaturation at 98°C for 10 s, annealing and elongation at 68°C for 11 min and final extension at 72°C for 10 min. The PCR products were purified and cloned using the Mighty TA-cloning Kit.

The deletion breakpoint was identified by primer walking.¹² First set of primers were set on intron 15 and intron 17b. The positions of both primers were shifted towards the putative breakpoint in steps of 300–600 bp. Finally, we obtained the 470–480 bp PCR product, which spans intron 15–intron 17b and

Figure 1 Cloning of the fragments of CFTR transcripts extracted from nasal swab. (a) Schematic diagram of the CFTR cDNA and domains of the CFTR protein. Eleven pairs of the primers were designed to amplify the overlapping segments as indicated. (b) Agarose gel electrophoresis of 11 reverse-transcriptase PCR products from the patient (P) and healthy control (C). The approximately 370 bp fragment amplified with the No. 4 pair of primers (arrow) lacked exon 9. The approximately 170 bp fragment amplified with the No. 8 pair of primers (arrow) lacked exons 16, 17a and 17b. (c) Sequencing results of the cloned 173 bp fragment revealed an aberrant CFTR mRNA lacking exons 16, 17a and 17b.

Table 2 Primer sequences for the analysis of the fragments of CFTR transcripts

No.	Position	Sequence 5'–3'	Fragment size (bp)
1	–53 F (exon 1) 562 R (exon 5)	TTAGGAGCTTGAGCCAGAC GGTTGTTGGAAGGAGACTAA	615
2	339 F (exon 4) 896 R (exon 7)	CAAGGAGGAACGCTCTATCG GCCTTCCGAGTCAGTTTCAG	558
3	814 F (exon 6b) 1369 R (exon 9)	GTTAAGGCATACTGCTGGGAA CAACCGCCAACTGTCCT	556
4	1010 F (exon 7) 1569 R (exon 10)	TCACCACCATCTCATTCTGC TGCTTTGATGACGCTTCTGT	560
5	1494 F (exon 10) 2038 R (exon 13)	GCCTGGCACCATTAAAGAAA TCCAGGAGACAGGAGCATCT	545
6	1933 F (exon 13) 2545 R (exon 14a)	ATGGGATGTGATTCTTTCGACC ATGTGTCCATGATGCTCACTG	613
7	2293 F (exon 13) 2961 R (exon 16)	AGGAGGCAGTCTGCTGAA CAGAAGGTCATCCAAATTGC	668
8	2860 F (exon 15) 3491 R (exon 19)	CATTCTGTTCTTCAAGCACCT AAGACTCGGCTCACAGATCG	632
9	3415 F (exon 18) 3937 R (exon 21)	ATCATGAGTACATTGCAGTGG GATCACTCCACTGTTTCATAGG	523
10	3810 F (exon 20) 4351 R (exon 24)	TGGTGTGCTCTGGGATTCAA GAAAGAGCTTCACCTGTCTG	542
11	4177 F (exon 23) 4649 R (exon 24)	GCTGATTGCACAGTAATTCTC ACTATTGCCAGGAAGCCATT	473

Abbreviation: CFTR, cystic fibrosis transmembrane conductance regulator.

harbors the breakpoint of the deleted sequence. The PCR product was sequenced directly. The established 'junction fragment' primers were used for the following study.

Amplification of the junction fragment

We developed a test to define the identified deletion by amplifying the junction fragment as shown in Figure 2b. The pair of 'junction fragment' primers: each on intron 15 (A) and on intron 17b (C) amplifies the deleted allele. The other pair of primers: both on intron 15 (A and B) amplifies the wild-type allele. The primer sequences are 5'-GGTCACAGGTGAGAGGAGGA-3' (A), 5'-TAA-CAGGTGAGGAGGCCAAG-3' (B) and 5'-TGAAGCTAGGACCCTTCACC-3' (C). The PCR protocol was polymerase activation at 94 °C for 5 min, followed by 30 cycles of PCR with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s and final extension at 72 °C for 5 min.

Quantitative fragment analysis by multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA), a quantitative PCR-based assay was performed to examine the presence of large deletion and duplication in the CFTR gene.¹³ We used the SALSA P091-C1 CFTR MLPA kit (MRC Holland, Amsterdam, The Netherlands). The kit contains probes for each of the 27 exons of CFTR. Fragments were separated on CEQ-8000 capillary electrophoresis system (Beckmann Coulter) using the Beckmann D1-labeled CEQ size standard 600.

RESULTS

Direct sequence of the CFTR gene following conventional PCR

None of the known CF-causing mutations was found in the CFTR gene of the patient. The patient had a genotype of 125G/C (c.-8G>C) heterozygote, 12/12 TG repeats (c.1210-34GT (9_13)) and 7/7 poly T (c.1210-12T (5_9)) in intron 8, M/M at p.M470V polymorphism. The frequencies of each polymorphism in healthy controls (96 alleles) are 6/96 (125C), 42/96 (TG12-T7) and 41/96 (470M).

Analysis of the fragments of CFTR transcripts from nasal swab

All the 11 pairs of primers (Figure 1a) successfully amplified the CFTR cDNA from the patient (P) and healthy control (C; Figure 1b). The healthy control had a genotype of 11/12 TG repeats and 7/7 poly T in intron 8.

PCR with the No. 4 pair of primers, which amplifies cDNA fragments spanning exons 7–10, produced the band of expected size (~560 bp) and the one of smaller size (~370 bp). The two bands were detected in both patient (P) and healthy control (C). Sequence

of the purified products revealed that the two bands correspond to the normal fragment and the one lacking exon 9 (data not shown).

PCR with the No. 8 pair of primers, which amplifies cDNA fragments spanning exons 15–19, produced the band of expected size (~630 bp) in both patient (P) and healthy control (C). However, another band of smaller size (~170 bp) was detected in patient (P) in addition to the normal band. The PCR product was purified and cloned. Sequencing results revealed that the shorter fragment (173 bp) lacked exons 16, 17a and 17b (Figure 1c).

Table 3 Primer sequences for the long-PCR analysis of CFTR transcripts

No.	Position	Sequence 5'–3'	Fragment size (bp)
1	–53 F (exon 1) 4649 R (exon 24)	TTAGGAGCTTGAGCCCAGAC ACTATTGCCAGGAAGCCATTT	4702
2	339 F (exon 4) 4649 R (exon 24)	CAAGGAGGAACGCTCTATCG ACTATTGCCAGGAAGCCATTT	4311
3	–53 F (exon 1) 2961 R (exon 16)	TTAGGAGCTTGAGCCCAGAC CAGAAGGTCATCCAAAATTGC	3013
4	81 F (exon 2) 2961 R (exon 16)	ATACAGACAGCGCTGGAAT CAGAAGGTCATCCAAAATTGC	2881
5	180 F (exon 3) 2961 R (exon 16)	GCTGGCTTCAAAGAAAATCC CAGAAGGTCATCCAAAATTGC	2782
6	3415 F (exon 18) 4649 R (exon 24)	ATCATGAGTACATTGCAGTGG ACTATTGCCAGGAAGCCATTT	1235
7	–913 F 2961 R (exon 16)	CCGGTAATTACGCAAGCAT CAGAAGGTCATCCAAAATTGC	3071
8	–786 F 2961 R (exon 16)	TTGGTCTTTTCTCCGACAC CAGAAGGTCATCCAAAATTGC	2944

Abbreviation: CFTR, cystic fibrosis transmembrane conductance regulator.

Analysis of the genomic rearrangement of the CFTR gene

Long PCR from exon 15 to exon 18 of CFTR detected approximately 4 kbp product from the patient genomic DNA and approximately 11 kbp product from the control genomic DNA. So-called primer walking was performed using the approximately 4 kbp product. We finally obtained the 470~480 bp product, which spans intron 15–intron 17b and harbors the breakpoint. Direct sequencing of the product revealed the deletion from nucleotide +1085 of intron 15 (the beginning of intron 15 defined as +1) to nucleotide +260 of intron 17b (the beginning of intron 17b defined as +1) with a loss of 7201 nucleotides, which was designated as c.2908+1085_3367+260 del7201 (Figure 2a).

PCR with the pair of 'junction fragment' primers (A and C in Figure 2b to amplify the deleted allele) amplified the 471 bp product from the patient genomic DNA (lane 1 in Figure 2c), but not from the control DNA (lane 2 in Figure 2c). PCR with the pair of 'normal fragment' primers (A and B in Figure 2b to amplify the wild-type allele) amplified the 477 bp product from both the patient DNA (lane 3 in Figure 2c) and the control DNA (lane 4 in Figure 2c). The data indicated that the patient has the heterozygous deletion of

Figure 2 Analysis of the genomic rearrangement of the *CFTR* gene. (a) Sequencing results of the junction fragment obtained from the genomic DNA by primer walking revealed the deletion from nucleotide +1085 of intron 15 to nucleotide +260 of intron 17b, with a loss of 7201 nucleotides. The numbers in the brackets correspond to the numbers of GenBank NT_007933.13. (b) Schematic diagram of the large 7201 bp deletion of the *CFTR* gene (hatched box). The location of the 'junction fragment' primers to amplify the deleted allele (A and C) and the other pair of primers to amplify the wild-type allele (A and B) is indicated. (c) Agarose gel electrophoresis of the 'junction fragment' (lane 1 and 2) and normal fragment (lane 3 and 4) from the patient (lane 1 and 3) and healthy control (lane 2 and 4).

c.2908 + 1085_3367 + 260del7201. The junction fragment was not identified in Japanese healthy controls ($n=48$).

Quantitative fragment analysis of the CFTR gene by MLPA

MLPA was performed to examine the presence of other large deletion or duplication in the CFTR gene. Figure 3a and b shows the original chromatograms of the patient (A) and the control (B). When the signals except for exons 16, 17a and 17b were normalized, the amount of the fragments corresponding to exon 16, 17a and 17b in the patient sample was reduced to 54, 50 and 54%, respectively, of the control sample. This finding confirmed the genomic deletion spanning exons 16, 17a and 17b in one allele. Genomic rearrangements were not detected in the other allele.

Long PCR analysis of the CFTR transcripts from nasal swab

Long PCR with the No. 1 pair of primers, which amplifies the full-length CFTR transcripts, produced a clear band of expected size (approximately 4700 bp) in a control subject (C), but a weak band of smaller size in the patient (P; Figure 4b). PCR of the fragments

spanning exons 4–24 (No. 2 pair of primers) produced the band of expected size (approximately 4300 bp) in both the patient (P) and control (C). Another band of smaller size (approximately 3800 bp) was detected in the patient (P). The smaller-size bands detected by No. 1 and 2 primers in the patient (P) likely correspond to the fragments which lack exons 16–17b (495 bp).

To examine the CFTR transcript from the other allele (without exons 16–17b deletion), PCR was carried out by setting the reverse primer on exon 16 (Figure 4c). PCR of the fragments spanning exons 1–16 (No. 3 pair of primers) produced a band of the expected size (approximately 3000 bp) in the control (C) but not in the patient (P). PCR of the fragments spanning exons 2–16 (No. 4 pair of primers) and those spanning exons 3–16 (No. 5 pair of primers) produced bands of the expected sizes (approximately 2900 and 2800 bp) in the control (C). However, the bands from the patient (P) sample were weak but of the same size. PCR of the fragments spanning exons 18–24 (No. 6 pair of primers) produced a band of the expected size in both patient (P) and healthy control (C). The density of the bands was similar. These data suggest that there is a deletion or skipping of exon 1 in the other allele.

Figure 3 Quantitative fragment analysis by MLPA. The original traces of samples from the patient (a) and control (b) using the SALSA P091-C1 CFTR MLPA kit. The signals corresponding to exons 16 (227.55 nt), 17a (255.94 nt) and 17b (282.51 nt) were reduced by approximately 50% in the patient sample (red arrows). The signals corresponding to exon 1 (152.73, 236.99 and 374.92 nt) were normal (blue arrows).

Figure 4 Long PCR analysis of the CFTR transcripts from nasal swab. (a) Six pairs of the primers were designed as indicated. (b, c, d) Agarose gel electrophoresis of the long-PCR products from the patient (P) and control (C). (b) PCR amplifying the transcripts from both alleles. No.1 pair of primers was designed to amplify the full-length cDNA. (c) PCR amplifying the transcripts from the other allele without exons 16–17b deletion. (d) PCR amplifying the 3'-end transcripts (exons 18–24) from both alleles. (e) Two pairs of the primers were designed to detect the splicing variants including alternative 5' exons (–1a and 1a).

Mouchel *et al.*¹⁴ reported alternative 5' upstream exons of CFTR (namely exons –1a and 1a), which were directly spliced to exon 2, excluding exon 1 from the transcript (Figure 4e). To test this possibility, PCR was carried out by setting the forward primer on exon –1a and the reverse primer on exon 16 (No. 7 and 8 pairs of primers, Table 3, Figure 4e). Two weak bands were detected in the control (C), but not in the patient (P; Figure 4c). Sequence of the purified product detected the splicing variants lacking exon 1 that exon –1a was spliced to exon 2 (data not shown). There was a 74bp insertion (part of intron 2) between exons 2 and 3 in the longer fragment. The data suggest that the CFTR transcript from the other allele (without exons 16–17b deletion) of the patient is not the reported splicing variant.

DISCUSSION

In the present study, we detected a large heterozygous deletion in the CFTR gene in a Japanese CF patient. Cloning and sequence analysis of the CFTR transcripts from nasal swab revealed a heterozygous deletion spanning exons 16, 17a and 17b. None of the CF-causing mutations was detected by conventional PCR, and direct sequence of all 27 exons, their boundaries and promoter region. The deletion was confirmed by MLPA and the direct sequence of the junction fragment obtained from the genomic DNA by primer walking, which revealed the mutation spanning intron 15 and intron 17b, c.2908 + 1085_3367 + 260del7201. The deletion was previously reported from a French CF patient and his healthy mother, who came from South Korea.⁹ The predicted CFTR protein lacks amino acids 970–1122, which correspond to the transmembrane regions 9, 10 and 11. A similar massive deletion spanning exon 16–17b: c.(?_2909)_(33367_?)del was found from a Japanese–Canadian patient with CF (Cystic Fibrosis Mutation Database), but the deletion breakpoint was different from the one in this study.

We also identified a novel splicing defect in the CFTR transcript from the other allele. The long PCR analysis of cDNA generated using

the gene-specific primer on exon 24 revealed a deletion/skipping of exon 1 in the CFTR transcript without exons 16–17b deletion. As neither CF-causing mutations nor genomic rearrangements were detected in this allele, the deletion/skipping of exon 1 is most likely caused by a splicing defect. Although exon 2 was conserved in the transcript, the exact breakpoint remains undetermined. Five different splicing mutations in intron 1 have been reported to the Cystic Fibrosis Mutation Database, but they were not detected in the present study. Mouchel *et al.*¹⁴ reported that the CFTR transcript lacking/skipping exon 1 was present at low levels in the human fetal lung. Alternative 5' upstream exons of CFTR were directly spliced to exon 2, excluding exon 1 from the transcript. Such splicing variants have not been reported in CF patients. We detected a small amount of the splicing variant lacking exon 1 in nasal swab from a control subject. However, the transcript lacking exon 1 from the patient was not this type of splicing variant. Though the exact breakpoint was unknown, the skipping of exon 1 would cause translation to start downstream, most likely utilizing an ATG in exon 4 of the CFTR transcript, which leads to the generation of the CFTR protein lacking the N-terminus.

CF is rare in Japan and only 80 cases have been counted since 1994 by the Research Committee of Intractable Pancreatic Diseases (data not published). The median survival time is about 18 years. Early diagnosis and treatment is important. The elevated sweat chloride because of CFTR dysfunction is the gold standard for CF diagnosis. The pilocarpine iontophoresis is the standard method to stimulate sweat-gland secretion in the forearm or back. However, it requires experienced personnel and a quality-controlled laboratory,¹⁵ which is hardly expected in Japan. Accordingly, detection of disease-causing mutations of CFTR is often necessary for the definite diagnosis of CF in Japan. Yoshimura and Anzai have found CFTR~~dele16–17b~~ mutation (the breakpoint was not examined) in three patients (all in homozygosis), although most of the other detected CFTR mutations

are of rare types and many of them have unknown or uncertain clinical relevance (Research Committee of Intractable Pancreatic Diseases, data not published). These data may suggest that the CFTR Δ 16–17b mutation is unique to Asian CF. Thus, we need to explore the presence of heterozygous deletion of 16–17b in Japanese CF patients. A ‘junction fragment’ test for the CFTR Δ 16–17b mutation was established in the present study (Figures 2b and c). This test requires only conventional PCR technique and can be included in the first-step analysis.

Even the extensive genomic analysis including the examination of intragenic rearrangements (by MLPA, and so on) was performed, 1–5% of CF alleles in Caucasians remains undetermined.⁴ The presence of mutations in a substantial number of Japanese CF patients remains unsolved (Research Committee of Intractable Pancreatic Diseases, data not published). Mutations in the regulatory elements located in sites distant from the coding regions are probably present in many of these cases, but they cannot be identified by currently available techniques.

The analysis of nasal swab transcripts can provide molecular evidence for dysfunctional CFTR in these cases. In the present study, we successfully detected the CFTR transcript lacking exons 16, 17a and 17b, and the other CFTR transcript lacking exon 1 in the extract of nasal swab obtained from the patient. This method has been used to detect aberrantly spliced transcripts and reduced mRNA levels that were associated with various types of nonsense, frameshift, stop and splicing mutations.^{16–19} In fact, we detected the CFTR transcripts with or without exon 9 in samples from both the patient and healthy control (Figure 1). The probability of alternative splicing of exon 9 is related to the genotype of poly T and TG repeats in intron 8.²⁰ The analysis of transcripts is not routinely performed to detect the CFTR mutations in medical genetic laboratories, but a recent study identified a novel insertion between exons 10 and 11.²¹ Another study of CF patients with only one mutation in the coding region of CFTR demonstrated a pathological reduction in the amount of RNA from the other allele in 5 of 11 patients.²² The respiratory mucosa in nasal cavities and bronchial airways has a similar structure. CFTR immunoreactivity was found in the apical membrane of ciliated epithelial cells and serous glandular cells of human nasal turbinates.²³ Nasal epithelial cells can be non-invasively and repeatedly obtained using a swab. Recent progress on reverse-transcriptase PCR allows amplification of mRNA starting with a low copy number. Deletion or insertion mutations as well as aberrant splicing of CFTR can be detected by the analysis of transcripts.

In conclusion, the analysis of CFTR transcripts from nasal swab was useful to detect a large heterozygous deletion of the CFTR gene and to identify a splicing defect in the transcript from the other allele in a Japanese CF patient. The CFTR Δ 16–17b mutation may be unique to Asian CF. Analysis of the CFTR transcripts from nasal swab is recommended, when conventional sequence analysis proves to be negative in Japanese CF.

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