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Notable contribution of large *CFTR* gene rearrangements to the diagnosis of cystic fibrosis in fetuses with bowel anomalies.

Alix de Becdelièvre¹, Catherine Costa¹, Annick LeFloch¹, Marie Legendre¹, Jean-Marie Jouannic², Jacqueline Vigneron³, Jean-Luc Bresson⁴, **Stéphanie** Gobin⁵, Josiane Martin¹, Michel Goossens¹ and Emmanuelle Girodon^{1*}

¹ Service de Biochimie-Génétique et Inserm U955 équipe 11, Groupe hospitalier Henri Mondor-Albert Chenevier, APHP, Créteil, 94010, France

² Service de Gynécologie Obstétrique, Hôpital Armand Trousseau, APHP, Paris, 75571, France

³ UF de Génétique, Service de Néonatalogie, Maternité A. Pinard, Nancy, 54042, France

⁴ Service de Génétique-Histologie-Biologie du développement et de la reproduction, Hôpital Saint-Jacques, Besançon, 25030, France

⁵ Département de Génétique, Laboratoire de Génétique moléculaire, Groupe hospitalier Necker-Enfants Malades, APHP, Paris, 75743, France

Running title: *CFTR* rearrangements in fetal bowel anomalies

*Author for correspondence:

Emmanuelle Girodon

Service de Biochimie Génétique

UF de Génétique

Groupe hospitalier Henri Mondor-Albert Chenevier, APHP

94010 Créteil

France

Tel : +33 1 49 81 48 22

Fax : +33 1 49 81 22 19

emmanuelle.girodon@inserm.fr

ABSTRACT

Grade III fetal bowel hyperechogenicity and/or loop dilatation observed at the second trimester of pregnancy can be due to several disease conditions, including cystic fibrosis (CF). Screening for frequent CF mutations is performed as a first step and, in certain situations, *e.g.* when a frequent CF mutation is found in the fetus, the increased risk of CF justifies an in-depth study of the second allele. To determine the contribution of large *CFTR* gene rearrangements in such cases, detected by using a semi-quantitative fluorescent multiplex PCR (QFM-PCR) assay, we collated data on 669 referrals related to suspicion of CF in fetuses from 1998 to 2009. Deletions were found in 5/70 cases where QFM-PCR was applied: dele19, dele22_23, dele2_6b, dele14b_15 and dele6a_6b, of which the last three remain undescribed. In 3/5 cases, hyperechogenicity was associated with dilatation and/or gallbladder anomalies. Of the total cases of CF recognized in the subgroup of first-hand referrals, deletions represent 16.7% of CF alleles. Our study thus strengthens the need to consider large *CFTR* gene rearrangements in the diagnosis strategy of fetal bowel anomalies, in particular in the presence of multiple anomalies.

KEY WORDS: CFTR, cystic fibrosis, deletions, rearrangements, fetal bowel anomalies, hyperechogenic bowel

INTRODUCTION

Fetal hyperechogenic bowel is a relatively common event detected at the second trimester ultrasound scan in 0.04–1.80% fetuses^{1,2}. It can be benign and transient, or an indicator of varied fetal pathologies including cystic fibrosis (CF) (MIM#219700), which has been reported in 2.5–10.0% of cases²⁻⁷.

CF is the most common severe autosomal recessive disorder in the Caucasian population with an overall incidence of about 1/3500 live births⁸⁻¹⁰. Although patients' life expectancy has greatly improved over past decades, CF remains a severe disease, allowing prenatal diagnosis when both parents are known carriers for CF mutations or when abnormal ultrasound signs are detected during pregnancy. It is now a European recommendation that mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene (OMIM*602421) should be investigated in fetuses with bowel anomalies such as **hyperechogenic** bowel and loop dilatation¹¹.

More than 1600 *CFTR* variations have been reported so far (<http://www.genet.sickkids.on.ca/cftr/>). Most are point mutations, with variable distribution and frequency according to ethnic/geographic origin¹². Large *CFTR* rearrangements like deletions, insertions or duplications, which can be identified by using semi-quantitative assays¹³⁻¹⁵, account for 1-3% of CF mutations and 14-26% of unidentified alleles in CF patients^{13,16,17}. To assess the frequency of large *CFTR* rearrangements in ultrasound signs suggestive of CF, data of 669 referrals related to suspicion of CF in fetuses were collated. We report here 5 cases out of 27 CF solved by using a semi-quantitative fluorescent multiplex PCR (QFM-PCR) assay.

SUBJECTS AND METHODS

Subjects

A series of 669 cases referred from different French clinics were investigated from 1992 to **October 2009** because of fetal bowel anomalies detected by routine ultrasound examination during the second or third trimester of pregnancy. Ultrasound signs were: hyperechogenic bowel (sonographic density greater than that of the surrounding bone), intestinal loop dilatation, intra-abdominal calcifications, meconium peritonitis and absence of **gallbladder**, each sign being isolated or combined. According to French legislation, informed consent was obtained from the parents during a genetic counseling session. Data from amniotic fluid digestive enzyme (AF-DE) activities ^{18,19} were collected, when available.

Samples from 450/669 couples were referred directly by clinicians to our laboratory (Group 1, first-hand referrals). DNA samples from the other 219 were referred for further investigation after screening for frequent mutations in another laboratory (Group 2).

***CFTR* gene analysis**

Genomic DNAs were extracted from whole blood samples collected on EDTA or from amniotic fluid using varied protocols. DNA concentration and quality were determined for each sample.

Screening for point mutations

Different study levels were carried out, depending on the clinical context ¹¹. As a first step, screening for frequent mutations was performed by using diverse commercial assays or, before 1998, by denaturing gradient gel electrophoresis (DGGE) ^{20,21} focused on at least 10 exons. According to patients' ethnic/geographic origins, complementary

screening for other mutations was carried out by using DGGE or denaturing high performance liquid chromatography (DHPLC) ²². In most cases no mutation was found and the couple was reassured. In cases where one mutation was identified, complete scanning of the 27 *CFTR* coding regions and a search for large rearrangements was carried out to look for a second CF mutation.

Screening for and characterization of large *CFTR* rearrangements

We used the QFM-PCR assay developed and implemented in our laboratory in 2004 ^{13,23}, retrospectively in certain cases referred before 2004. Abnormal patterns were confirmed by at least one other QFM-PCR experiment. Real-time quantitative-PCR (TaqManTM) was performed in one case. Characterization of the rearrangement breakpoints was achieved by a combination of long-range PCR and sequencing experiments.

RESULTS

In Group 1 of first-hand referrals, 408/450 couples (91%) were tested for frequent mutations according to their geographic origin. Comprehensive *CFTR* gene studies were carried out in 42 cases, including a search for large rearrangements in 17 of them (Table 1). CF was confirmed in 9 fetuses (2.0% of 450 fetuses), including 2 with a deletion (cases 1 and 4).

In Group 2 of second-hand referrals composed of 219 couples, 101 cases were referred for complementary screening depending on geographic origin, 92 because one frequent mutation was identified, 10 because of suggestive ultrasound signs and 16 because of technical problems. Search for large rearrangements by QFM-PCR was undertaken in

53 cases (Table 1). CF was confirmed in 18/219 cases (8.2%), including 3 with a large deletion (cases 2, 3 and 5).

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Overall, of the 70 cases screened for gross rearrangements, a partial gene deletion was characterized in 5 (7.1%) (Tables 2 and 3). Large *CFTR* deletions represent in this study 11.1% (6/54) of alleles in the 27 CF fetuses: 16.7% (3/18) in the 9 CF fetuses of Group 1 and 8.3% (3/36) in the 18 CF fetuses of Group 2.

Case 1

First cousins of Turkish origin were referred for fetal hyperechogenic bowel at 20 weeks of gestation²⁴. Screening for frequent mutations by the CF-OLA assay was negative in both parents, while 3 peaks corresponding to exon 19 were missing in the fetus. The deletion was confirmed by real-time quantitative-PCR (TaqManTM), in heterozygosity in the parents and homozygosity in the fetus. Given the late stage of gestation, the pregnancy was continued to term. The 10-year-old girl has classical CF with a positive sweat test (90 mEq/L).

Case 2

The couple was referred at 20 weeks because of fetal bowel hyperechogenicity and dilatation associated with non visualization of gallbladder. Screening for frequent mutations in another laboratory identified W1282X in the father and the fetus. Complementary analysis led to the identification of a new heterozygous 55kb deletion in the mother and the fetus, encompassing exons 2 to 6b. An 11-bp sequence homology overlapped the inserted sequence and the 3' breakpoint. The genotype was compatible with classical CF and, given the persistence of ultrasound signs, the parents chose to terminate the pregnancy at 26 weeks.

Case 3

The couple was referred at 30 weeks for fetal **ascites**, **hyperechogenic bowel wall with hypoechogenic loop content, which is indicative of incipient loop dilatation**, microcolon and gallbladder sludge. The father and the fetus were found F508del heterozygous in another laboratory. Abnormally high AF-DE activities were compatible with **ascites**, probably caused by meconial peritonitis. We identified a heterozygous previously described deletion of exons 22 and 23 in the mother and the fetus ¹⁴. Termination of pregnancy was performed and the diagnosis of CF confirmed by fetopathological examination.

Case 4

The couple was referred at 25 weeks because of non visualization of fetal gallbladder associated with moderate digestive dilatation and hyperechogenicity. As screening for frequent mutations according to their geographic origin was negative in both parents, the study was stopped and the pregnancy carried out to term. However, neonatal screening for CF by determination of immunoreactive trypsinemia was positive in the baby, as was the sweat test (97 mEq/L). Complete analysis of the *CFTR* gene led to identification of the baby as compound heterozygous for the rare 2347delG mutation, inherited from the mother, and a paternal new large deletion removing exons 14b and 15 (Figure 1).

Case 5

The couple was referred at 32 weeks because of fetal **ascites** and hydramnios. The father and the fetus were found F508del heterozygous in another laboratory. A novel deletion of exons 6a and 6b was identified by QFM-PCR in the mother and the fetus. Further characterization showed that it retains the first 12 codons of exon 6a, with a 7bp

homology at the breakpoints in exon 6a and intron 6b. Given the late stage of pregnancy, it was continued to term and the mother gave birth to a CF baby.

DISCUSSION

This is the first study reporting on the occurrence and frequency of large *CFTR* rearrangements in cases of bowel anomalies, hereby demonstrating their notable contribution to the diagnosis of CF. The frequency of gross deletions among CF alleles found in affected fetuses (16.7% of first-hand referrals), which appears much higher than the 1.3% found in postnatally diagnosed CF patients in the same geographic population ¹³, makes our QFM-PCR assay the method of choice for 2nd line screening after testing for frequent mutations, especially in emergency cases. With the development of commercial kits such as the Multiplex ligation-dependent probes amplification kit (MLPA[®], MRC Holland, Amsterdam, the Netherlands) and the implementation of laboratory networks for the molecular diagnosis of CF ¹¹, the frequency of *CFTR* gross rearrangements could even be re-evaluated upwards in the next few years.

4 We here refer to 5 large deletions, all of which could be detected using the MLPA[®] assay, including 3 as yet undescribed: dele2_6b, dele6a_6b and dele14b_15.

As for other rearrangements described ^{13,16}, these deletions seem to result from non-homologous recombination, with short sequence homologies at the breakpoints. Such deletions can result from slipped mispairing during DNA replication ¹⁴. The “super hotspot” CCAAR, described in breakpoints of *CFTR* large rearrangements ²⁵ was however not found in any of the five deletions reported in our study. Although the consequences of the deletions have not been studied at the functional level, it is

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presumed they truncate the CFTR protein and can thus be considered to have a severe effect. Identification of deletions and consecutive confirmation of the diagnosis of CF had implications for genetic counseling in the families, since it enabled prenatal diagnosis to be offered in further pregnancies as well as cascade testing in relatives.

The clinical presentations of these 5 cases were representative of the signs suggestive of CF in fetuses ⁷. Of note, **3/5 CF fetuses carrying a deletion had associated digestive signs, all 3 with gallbladder anomalies**. Few studies have been published about the association between CF and fetal gallbladder anomalies ^{7,26,27}, thus calling for larger studies to assess better the frequency of CF in fetuses with such anomalies.

The present cases also further illustrate the variety of patterns of AF-DE activities. As recently evidenced in a wide retrospective study, AF-DE could still be of value for prenatal diagnosis of CF, though, after **20** weeks, a physiological low value may be impossible to distinguish from a pathological one ²⁸.

In conclusion, our study strengthens the need to consider large *CFTR* gene rearrangements in the diagnosis strategy of fetal bowel anomalies, in particular in the presence of multiple anomalies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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TITLE AND LEGEND TO THE FIGURE

Figure 1. Identification and characterization of 2751+1355_3040+243del7613bp (dele14b_15, case 4).

A. Electropherogram from a QFM-PCR experiment in fetus 4 (blue), carrying a deletion of exons 14b and 15 at the heterozygous state (indicated by arrows), superimposed with that of a normal control (red). The deletion is evidenced by a 2-fold decrease in fluorescence intensity in the patient as compared to the normal control. The x-axis represents the computed length of the PCR products in base pairs as determined using an internal lane standard (green). The y-axis shows fluorescent intensities in arbitrary units. Normalization of the profiles was performed using *F9* (chromosome X) or *DSCR1* (chromosome 21, not seen in this window) reference genes¹³. Presence of double peaks at exon 9 corresponds to heterozygosity at the intron 8 poly(TG)-polyT locus.

B. The breakpoint junctions of the deletion, as determined by direct sequencing.

C. Sequence comparison and alignment between the recombined region (dele14b_15) and the wild-type sequences spanning the 5'- (IVS14a) and 3'- (IVS15) breakpoints. Identical sequences are in black and the direct repeat (GTGGG-CTG) is indicated underlined.

Table 1. Reasons of screening for large rearrangements

In Group 1 (first-hand referrals): 17/450
<ul style="list-style-type: none"> - First step of the study: one CF mutation identified (n=8) <i>F508del (n=6), 394delTT (n=1), Q1352H (n=1)</i> - Abnormal AF-DE (n=4) - Consanguinity in the couple (n=1) - Very suggestive ultrasound signs* (n=4)
In Group 2 (second-hand referrals): 53/219
<ul style="list-style-type: none"> - First step of the study: one CF mutation identified in another laboratory (n=45) <i>F508del (n=36), N1303K (n=3), G542X (n=2), G551D, R553X, W1282X, 3849+10kbC>T (n=1 for each)</i> - Abnormal AF-DE (n=1) - Consanguinity in the couple and presence of the [R74W;V201M;D1270N] complex allele (n=1) - Very suggestive ultrasound signs* (n=6)

*Very suggestive ultrasound signs meant several abnormal signs were associated and/or clinicians insisted on a comprehensive study of the *CFTR* gene.
AF-DE: amniotic fluid digestive enzymes

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Table 2. Phenotype and genotype data in fetuses carrying at least one large *CFTR* deletion

Case	Year	Gestational of the age (weeks) study	Ultrasound findings					Amniotic fluid digestive enzymes	Outcome	Allele 1 ^a	Allele 2 (short name)	Geographic origin for the deletion
			HB	LD	GB	Ascites	Other					
1	1998	20	+	-	-	-	-	Normal	Birth, CF	dele19	dele19	Turkey
2	2004	20	+	+	NV	-	-	Abnormally low	TOP	W1282X	dele2_6b	Denmark
3	2005	30	+	-	Sludge	+	microcolon	Abnormally high	TOP	F508del	dele22_23	France
4	2008	25	+	+	NV	-	-	NP	Birth, CF	2347delG	dele14b_15	Brittany/ Germany
5	2009	32	-	-	-	+	hydramnios	NP	Birth, CF	F508del	dele6a_6b	Portugal

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HB: hyperechogenic bowel; LD: loop dilatation; GB: gallbladder; NV: not visualized; TOP: termination of pregnancy.

^a Mutation names were given according to the international consortium mutation database (www.genet.sickkids.on.ca/cftr). For mutations denominated at the nucleotide level, the A of the ATG translation start codon was numbered as +133, in accordance with the current *CFTR* gene numbering based on cDNA sequence (GenBank NM_000492.3).

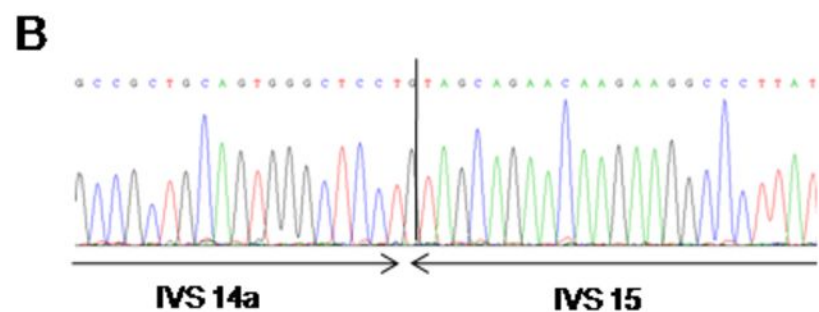
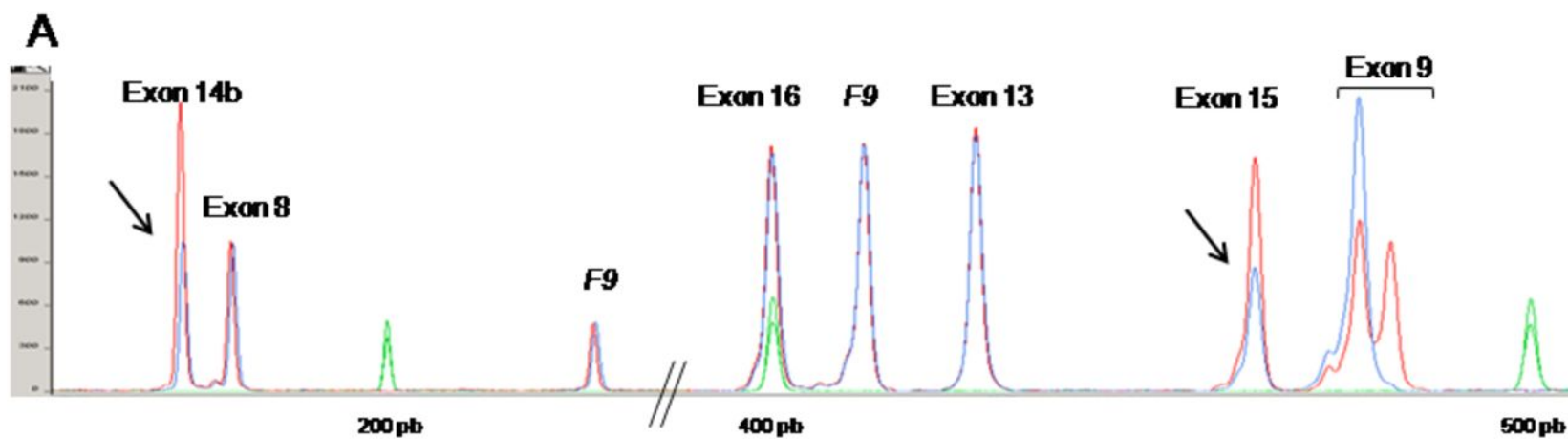
Table 3. Genotype data of the deletions found in CF fetuses

Case	Allele 1 ^a	Allele 2 ^a	Short name of the deletion	Motif sequence at the breakpoints
1	3601-2880_3849+2150del5279bp ^b (c.3469-2880_3717+2150del5279bp)		dele19	AACT (direct)
2	W1282X	185+2909_1002-1620del55429ins17bp ^c (c.53+2909_870-1620del55429ins17bp)	dele2_6b	CAGCTCTAGTT (direct)
3	F508del	IVS21-78_IVS23+577del1532bp (c.3964-78_4242+577del1532bp)	dele22_23	ACT (direct)
4	2347delG	2751+1355_3040+243del7613bp (c.2619+1355_2908+243del7613bp)	dele14b_15	GTGGGCTCCTG (5') and GTGGGACTG (3') (direct)
5	F508del	746_1002-1547del3273bp (c.614_870-1547del3273bp)	dele6a_6b	TCCTTTG (inverted)

^a Mutation names were given according to the international consortium mutation database (www.genet.sickkids.on.ca/cftr). For mutations denominated at the nucleotide level, the A of the ATG translation start codon was numbered as +133, in accordance with the current CFTR gene numbering based on cDNA sequence (GenBank NM_000492.3). Deletion names according to the HGVS nomenclature (the A of the ATG translation start codon being numbered as +1) are indicated in brackets.

^b Fetus homozygous for the deletion, initially reported as 3600+15kdel5.3kb ²⁴

^c 17bp insertion consists of GTACTCAACAGCTCTAG



C

ivs14a	AGTGGGCTCCTGCGTGGCCCAAGCCT
dele 14b_15	AGTGGGCTCCTGTAGCAGAACAAGAA
ivs15	TTGGTGGGACTGTAGCAGAACAAGAA

Figure 1