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Brief Communications

Amniotic Fluid Digestive Enzyme Analysis Is Useful for Identifying *CFTR* Gene Mutations of Unclear Significance

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BACKGROUND: The large number of *CFTR* [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)] mutations and the existence of variants of unclear significance complicate the prenatal diagnosis of cystic fibrosis (CF). The aim of this study was to determine whether the pattern of amniotic fluid digestive enzymes (AF-DEs) could be correlated with the severity of *CFTR* mutations.

METHODS: The AF-DE pattern (γ-glutamyltranspeptidase, aminopeptidase M, and the intestinal isoform of alkaline phosphatase) was retrospectively analyzed in 43 AF samples. All fetuses presented 2 *CFTR* mutations, which were classified according to the severity of the disease: CF/CF (n = 38); CF/*CFTR*-related disorders (n = 1); and CF/unknown variant (n = 4). The relationships between clinical CF status, *CFTR* mutations, and AF-DE pattern were studied.

RESULTS: Of 38 severely affected CF fetuses, an "obstructive" AF-DE pattern was observed in 15 of 15 samples collected before 22 weeks, irrespective of the *CFTR* mutation (diagnostic sensitivity, 100%; diagnostic specificity, 99.8%). In the 23 fetuses evaluated after 22 weeks, the AF-DE pattern was abnormal in 7 cases and noncontributive in 16 (diagnostic sensitivity, 30.4%; diagnostic specificity, 99.8%). Of the 5 questionable cases (F508del/N1224K, F508del/L73F, 3849+10kbC>T/G1127E, F508del/S1235R, F508del/G622D), all were CF symptom free at 2–4 years of follow-up. The AF-DE pattern (<22 weeks) was typical in 3 cases but abnormal in the last 2 cases.

CONCLUSIONS: AF-DE analysis is of value for prenatal CF diagnosis in classic forms of CF and could be helpful in nonclassic CF.

Cystic fibrosis (CF)[7] is the most common autosomal recessive disorder in the Caucasian population *(1)*. Although life expectancy has greatly improved, CF remains a severe disease, and the number of requests for prenatal diagnosis is high. Prenatal diagnosis has consisted of assessing amniotic fluid digestive enzyme (AF-DE) activities at 17 weeks' gestation *(2, 3)*. AF-DE patterns are related to the physiological maturation of the fetal digestive tract. At 11–12 weeks, opening of pharyngeal and anal membranes coincides with the massive arrival of intestinal microvillar enzymes in the AF. After 18 weeks, maturation of the anal sphincter impairs this flow. In CF-affected fetuses, hyperviscosity of intestinal secretions causes AF-DE activities to be low at 16 weeks' gestation ("obstructive" pattern). After 22 weeks, it is impossible to distinguish a physiological low value from an "obstructive" one, but bile vomiting or anal leakage can be observed, reflecting meconium ileus.

Cloning of the *CFTR* gene [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette subfamily C, member 7)] has allowed molecular prenatal diagnosis of CF *(4)*. Besides the main mutation (F508del)*(5)*, more than 1600 other mutations have been reported (http://www.genet.sickkids.on.ca/cftr/app), leading to a large spectrum of phenotypes, ranging from classic CF to monosymptomatic or CFTR-related disorders (CFTR-RDs), such as congenital bilateral absence of the vas deferens or chronic pancreatitis. Mutations can be considered to be CF-causing, CFTR-RD–causing, of neutral effect *(6)*, or of unknown clinical relevance [unclassified variant (UV)].

Prenatal CF diagnosis may be offered to couples identified through familial screening *(7)* or after the detection of fetal bowel anomalies during routine ultrasound scanning. Identification of 1 UV or 1 CFTR-RD mutation makes genetic counseling difficult. The aim of this study was to establish the value of AF-DE assay in such cases.

Our database consisted of 43 fetuses carrying 2 *CFTR* mutations or variants, from whom AF was sampled at 16–33 weeks of gestation during the period 1984–2008. Fourteen fetuses had a family history of CF, and 29 had bowel anomalies detected during rou-

[7] Nonstandard abbreviations: CF, cystic fibrosis; AF-DE, amniotic fluid digestive enzyme; CFTR-RD, cystic fibrosis transregulator–related disorders; GGTP, γ-glutamyltranspeptidase; AMP, aminopeptidase M; iALP, intestinal isoform of alkaline phosphatase.

tine ultrasound scanning *(8)*. Informed consent for AF sampling and biochemical and genetic testing was obtained from all patients. In France, informed consent is adequate for samples collected as part of routine care, and local ethics committee approval is not required. AF-DEs, including γ-glutamyltranspeptidase (GGTP), aminopeptidase M (AMP), and the intestinal isoform of alkaline phosphatase (iALP) were assayed *(3, 9)*. Results were expressed in percentiles previously defined for 10 000 control cases. Three abnormal patterns were defined: (*a*) between 16 and 22 weeks, an "obstruction" pattern characterized by all enzyme activities below the first percentile; (*b*) after 22 weeks, "bilious vomiting" pattern characterized by GGTP and AMP activities >99th percentile while the iALP values are typical; and (*c*) after 22 weeks, "anal leakage" characterized by all enzyme values >99th percentile. All 3 patterns were observed in 0.2% of control cases.

Genetic analysis was performed with either a technique aimed at detecting the most frequent mutations or techniques aimed at detecting unknown mutations, such as denaturing gradient gel electrophoresis, denaturing HPLC, or sequencing analysis *(10)*. Mutations were checked against parental DNA for each propositus. Mutations were classified according to their presumed clinical relevance *(6, 10)*: CF-causing mutations, CFTR-RD mutations, and UVs. Fetuses were classified into 3 groups according to their genotype: (*a*) CF/CF (n = 38), (*b*) CF/CFTR-RD (n = 1), and (*c*) CF/UV (n = 4).

CF status (classic form of CF or CF symptom free) was defined either in a fetal pathology examination by the presence of meconium ileus or after birth by biochemical tests (immunoreactive trypsin and sweat testing) and upon clinical presentation. Correlations between CF status, *CFTR* mutations, AF-DE pattern, and ultrasound signs were studied in each group. Clinical outcome was used to define 2 groups: CF affected (38 cases) and CF symptom free (5 cases).

CFTR genotypes and outcomes for CF-affected fetuses are presented in Table 1. All 38 cases presented with 2 CF mutations that were presumed to be of severe effect, including the rare 297−3C>T initially identified in a CF patient with pancreatic sufficiency but who died at age 44. This mutation was considered a CF splicing defect on the basis of mRNA studies *(11)*. Pregnancy was terminated at the parents' request in 34 cases. An abnormal digestive tract (meconium ileus) was observed in 33 cases (autopsy refused in 1 case). Pregnancy went to term in 4 cases, and the children presented with severe CF symptoms.

Before 22 weeks, an "obstructive" AF-DE pattern was observed in 15 cases, irrespective of the *CFTR* mutation (7 F508del homozygotes and 8 compound heterozygotes), giving a 100% diagnostic sensitivity and a 99.8% diagnostic specificity. After 22 weeks, AF-DE pattern was noncontributive in 16 cases and abnormal in 7 cases ("anal leakage" in 6, "bile vomiting" in 1), which consisted of 4 F508del homozygotes and 3 compound heterozygotes, giving a 30.4% diagnostic sensitivity and a 99.8% diagnostic specificity.

Of the 38 CF-affected fetuses, 9 were from at-risk families, and 29 received their diagnosis after routine ultrasound scans. The most frequent sign was hyperechogenic fetal bowel (n = 22), which was isolated in 12 cases and associated in 10 cases with nonvisualization of the fetal gall bladder (n = 7), dilated loops (n = 2), and both signs (n = 1). The other bowel anomalies were peritonitis (n = 3), nonvisualization of the fetal gall bladder (n = 2), and dilated loops (n = 2). In the 9 fetuses from at-risk families, the second-trimester ultrasound scan detected a hyperechogenic fetal bowel in 1 case and no anomaly in 1 case; ultrasound scanning was not performed in the other 7 cases (i.e., termination of pregnancy before ultrasound scan). Also among these 38 cases were 8 fetuses that had both the AF-DE assay before 22 weeks and an ultrasound scan. All 8 fetuses presented with an "obstructive" AF-DE pattern, and 7 had an abnormal ultrasound scan. After 22 weeks, of the 23 cases studied because of an abnormal ultrasound scan, 8 (34.8%) had an abnormal AF-DE pattern.

Of the 43 cases total, 5 were questionable and were studied because of the discovery of 2 *CFTR* mutations at parental screening. Four of these cases had a severe CF mutation associated with a UV. Pregnancy went to term in all cases, and the infants (2–4 years old) are CF symptom free. None of these variants had previously been described, and their potential for causing disease was unknown. The fifth case (F508del/S1235R) also presented with a prenatally diagnosed DiGeorge syndrome (cardiac malformation) with an imperforate anus detected at birth (29 weeks). Amniocentesis was performed before 22 weeks gestation in all 5 cases. An "obstructive" AF-DE pattern was observed in 2 cases (related to the imperforate anus in the F508del/S1235R case). A nonpathologic pattern was observed in the 3 others. None of these 5 cases presented with CF-related abnormal ultrasound signs.

In the 1980s it was established that performing the AF-DE assay before 22 weeks allowed prenatal diagnosis of CF *(2, 12)*. After the identification of *CFTR* mutations, however, the correlation between mutations and AF-DE pattern was not studied because most families underwent genotyping that used chorionic villi *(13)*. In this series, we observed a 100% correlation between an "obstructive" AF-DE pattern and severe CF, irrespective of the mutation, provided that the AF is sampled between 16 and 22 weeks. The abnormal patterns observed in CF reflect meconium ileus and are not observed in CF carriers; however, these patterns are

Table 1. ***CFTR*** **genotypes and outcomes in cases of fetuses affected by severe CF and in 5 questionable cases.**

CFTR mutation	Cases, n	Outcome/follow-up
CF/CF mutation (n = 38)		
F508del/F508del	21	TOP[a] (n = 20); birth, severe CF (n = 1)
F508del/unidentified severe mutation[b]	3	TOP (n = 3)
F508del/G551D	2	TOP (n = 2)
F508del/4005+1G>A	1	TOP
F508del/2711delT	1	Birth, severe CF
F508del/297−3C>T	1	TOP
F508del/3120+1G>A	1	TOP
F508del/405+1G>A	1	TOP
F508del/711+1G>T	1	TOP
F508del/Q1042X	1	TOP
F508del/dele22−23	1	TOP
F508del/2789+5G>A	1	Birth, severe CF
dele19/dele19[c]	1	Birth, severe CF
W1282X/dele2−6b	1	TOP
1078delT/394delTT	1	TOP
CF/unknown variant (n = 4)		
F508del/G622D	1	Birth, no clinical sign of CF
F508del/N1224K	1	Birth, no clinical sign of CF
F508del/L73F	1	Birth, no clinical sign of CF
3849+10kbC>T/G1127E	1	Birth, no clinical sign of CF
CF/CFTR-related disorder (n = 1)		
F508del/S1235R	1	Birth, no clinical sign of CF (del22q11 + imperforate anus)[d]

[a] TOP, termination of pregnancy.
[b] Severe CF in siblings.
[c] Parents were first cousins.
[d] del22q11 is DiGeorge syndrome.

not specific to CF and can be observed in fetal digestive tract malformations, cytomegalovirus infection, or fetal distress *(14)*. Nonetheless, because these abnormal patterns are very rare in typical pregnancies, their presence in families at risk of CF is suggestive of severe CF.

In families not at risk, fetal bowel anomalies usually lead to *CFTR* gene screening. Previous studies have revealed a 3%–5% prevalence of CF in fetuses with hyperechogenic fetal bowel, leading to 10%–13% residual risk of CF when 1 *CFTR* mutation is detected *(8, 15)*. An abnormal AF-DE pattern would increase the risk of fetal CF, leading to a complete *CFTR* gene screening. A typical AF-DE pattern, when observed before 22 weeks, would exclude severe CF.

Prenatal CF diagnosis is particularly complicated when a severe CF mutation is associated with a CFTR-RD mutation or a novel mutation *(6)*. Could AF-DE assay be helpful? Such a situation was observed in 5 cases of our series. In 3 cases (N1224K, L73F, and G1127E), the AF-DE pattern was typical. The children (2, 3, and 3 years old, respectively) did not present clinical signs of CF. F508del was in trans of N1224K and L73F, and the variable CF-causing 3849+10KbC>T was in trans of G1127E *(16)*. An "obstructive" AF-DE pattern observed in the F508del/S1235R fetus was in fact explained by DiGeorge syndrome (imperforate anus) *(14)*. The 4-year-old girl has not demonstrated any clinical signs of CF. S1235R was recently considered a CFTR-RD mutation or a neutral variant *(10)*. In the last case (F508del/G622D), we have no clear explanation for the abnormal AF-DE pattern. The 4-year-old infant presented a typical immunoreactive trypsin result at birth, a typical sweat test result, and a typical clinical course; however, we cannot rule out the late development of a CFTR-RD. The G622D variant, identified in infertile patients, was found at an allelic frequency of 0.18% in a population of African Americans *(17)*. Its clinical significance remains unclear, although

functional studies have demonstrated a deleterious effect *(18)*. One hypothesis is that the G622D phenotype is clinically expressed in intestinal microvilli only during fetal life and is silent thereafter. Another explanation is that it delays the physiological permeation of anal membranes and thus may mimic an "obstructive" pattern. This observation could be related to the finding of so-called "mild" mutations in neonates with increased immunoreactive trypsinemia *(19)* and fetuses with fetal bowel anomalies *(20)*.

Our results show that a typical AF-DE pattern before 22 weeks excludes severe CF disease and that this observation can be helpful in cases of questionable *CFTR* genotypes. It would be of interest to further investigate AF-DE patterns in a prospective multicenter study.

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