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PRENATAL DIAGNOSIS OF CYSTIC FIBROSIS BY MICROVILLAR MEMBRANE ENZYME ANALYSIS IN AMNIOTIC FLUID

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Prenatal diagnosis was performed in 92 pregnancies high-risk for cystic fibrosis during six years. Amniotic fluid samples obtained by amniocentesis were examined with regard to their microvillar membrane enzyme activity. Though trehalase, alkaline phosphatase isoenzymes and L-gamma-glutamyltransferase in the amniotic fluid are not specific markers of cystic fibrosis, their activity is significantly lower than in normal pregnancies. By measuring the three enzymes simultaneously, sensitivity, specificity and reliability of the method were found to be over 92 %. It is concluded that mid-trimester amniotic fluid diagnosis is indispensable for some heterozygotic couples for cystic fibrosis even in the possession of DNA (desoxyribonucleic acid) methods.

INTRODUCTION

Since 1983, the use of amniotic fluid microvillar membrane enzyme analysis for the prenatal diagnosis of cystic fibrosis (CF) has been suggested by several laboratories. The most important enzymes are: peptidases, e.g., L-gamma-glutamyltransferase (GGT), disaccharidases (DS), (e.g., trehalase, lactase) and phosphatases, e.g., alkaline phosphatase (ALP). Since the glandular cells of affected organs (sweat glands, pancreas, intestines, bronchi) are rich in microvilli, it is not surprising that in the case of fetal CF, microvillar enzyme activity in the amniotic fluid is lower than

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in normal pregnancies /2, 5, 10, 14/.

We described our results with this method first in 1984, then in the following year /13, 14/. During the past 6 years we have had a sufficient number of cases to evaluate retrospectively and determine the place and value of amniotic fluid enzyme analysis, besides molecular genetic methods, in the prenatal diagnosis of CF.

MATERIALS AND METHODS

Between 1 January, 1983 and 31 December, 1988, amniocentesis was performed in the 16-21 gestational weeks in 92 pregnancies because of high risk for CF (25 %) or pathological ultrasound findings (dilated intestinal lumen and/or meconium plug) at the Genetic Counselling Unit of the Department of Obstetrics and Gynecology, Debrecen.
The enzyme analysis of amniotic fluid samples taken from these 92 and from 175 healthy pregnancies of the same gestational age was done by the following methods:

1. Determination of the activity of two disaccharidases (trehalase and lactase) (see 14).
2. Kinetic determination of alkaline phosphatase (ALP) activity. The reagent contained: 0.60 ml buffered substrate (1.02 mol/l dietanolamin-HCL, pH 9.8, 10.27 mmol/l p-nitrophenylphosphate-chlorid, 0-5 mmol/l $MgCl_2$ and 60 ul amniotic fluid supernatant. Enzyme activity was determined from p-nitrophenol released after a 3-minute incubation period by photometry at 405 nm.
2. Measurement of enzyme activity to determine ALP isoenzyme activity in the presence of 5.0 mmol/l L-phenylalanine (a placental and intestinal isoenzyme inhibitor) was performed as described above.

$$\text{Inhibition ratio (\%)} = 100 - \frac{\text{activity in the presence of inhibitor}}{\text{ALP activity}} \times 100$$

4. L-gamma-glutamyltransferase activity (GGT) was measured by Merckotest 14302 gGT (FRG) kinetic kit. The reaction mixture contained: 1.0 ml buffered substrate (110 mmol/l of TRIS, pH 8.25, 110 mmol/l of glycilglycin, 4.4 mmol/l of L-gamma-glutamyl-3-carboxy-4-nitroanilid and 20 ul of amniotic fluid supernatant. After a 1-minute lag phase, increase in absorption was recorded every minute during a 3-4 minute duration. Enzyme activity was expressed in volume activity (U/l).

To discriminate "affected" (P1) from "healthy" groups (P2), we have found a value designated by Xcrit, which falls between

the average of the two groups. If the enzyme value was greater than the Xcrit, the patient was judged healthy, if it was lower, he/she was judged affected. This Xcrit value was chosen according to the principle of "maximum likelihood".

Let the P1 population be of normal distribution with m_1 expected value and sl standard deviation, and similarly the P_2 population with the parameters m_2 and s_2, where $m_2 > m_1$. In this case, the likelihood function is:

$$L_1(x) = \frac{1}{\sqrt{2\pi} \cdot s_1} \cdot e^{-\frac{(x - m1)^2}{2s_1}}$$

for the P1 population and

$$L_2(x) = \frac{1}{\sqrt{2\pi} \cdot s_2} \cdot e^{-\frac{(x - m_2)^2}{2s_2}}$$

for the P2 population.
A patient having a given x enzyme value is considered healthy if

$$L_2(x) > L_1(x).$$

After the necessary algebraic transformations we have the following quadratic unequality:

$$x^2 \left(\frac{1}{s_1^2} - \frac{1}{s_2^2}\right) - 2x \left(\frac{m_1}{s_1^2} - \frac{m_2}{s_2^2}\right) + \frac{m_1^2}{s_1^2} - \frac{m_2^2}{s_2^2} - 2 \cdot \log \frac{s_2}{s_1} > 0$$

If $s_1 = s_2$, then Xcrit = $(m_1 + m_2)/2$, if $s_2 > s_1$, then Xcrit will have 2 values, one of which is smaller than m_1, and if $s_1 > s_2$, then one of the Xcrit values is greater than m_2, but in the two latter cases one of the solutions is smaller than m_2. If the Xcrit value falls between m_1 and m_2, this value is accepted, and if it is below m_1 or above m_2, distinction is not possible from a clinical point of view (it occurs when m_1 and m_2 are very close to each other, which is significantly different in our material).

The means and standard deviation were used in computing the data.

RESULTS

For data evaluation, the amniotic fluid samples were classified into three groups. The first group included samples of fetuses with CF. The second group consisted of healthy infants of heterozygotic parents for CF. The theoretical

probability of these being heterozygotic was 2/3. The third group was the control group, where amniotic fluid samples were taken from pregnancies unrelated to high risk for CF. The number of cases was not identical in each group, ALP and GGT examinations were introduced later.

The median values of enzyme activity in the amniotic fluid samples of healthy infants in the 16-18 gestational weeks are:

Enzyme	16th week	18th week	Unit
Trehalase	1.22	0.71	U/g protein
ALP	16.81	20.87	U/l
Inhibition (%)	76.64	71.14	%
GGT	269.21	171.51	U/l

There is a significant difference between the affected and healthy groups for each of the enzymes ($p < 0.0001$). It means that the selected enzymes are very good markers, even if not all are of specifically intestinal origin.

The values of enzyme activity - considering the means of the control group as median - were counted in MoM (multiple of the median) units (Table I). Table I shows that in relation with the control group, enzyme activity values in the affected group are around 0.5 MoM, while activity values of the 2/3 CF-heterozygotic group are practically identical to those of the normal cases.

In distinguishing normal and affected populations we were looking for a value, which - according to the principle of "maximum probability", and the normal distribution curve would be the most suitable for the discrimination between the two groups and which can be used for classifying the enzyme values into affected and healthy groups /14/.
Our Xcrit results were:

Trehalase = 0.832 MoM
ALP = 0.817 MoM
Inhibition (%) = 0.808 MoM
GGT = 0.621 MoM

TABLE I

Microvillar membrane enzyme activity in amniotic fluid samples in 15 - 20 weeks of gestation
(Values in MoM, 95 % confidence limits in brackets)

Enzyme	CF-affected	2/3-heterozygotic	Control
Trehalase	n = 42 0.445±0.168 (0.278-0.613)	n = 52 0.928±0.163 (0.765-1.091)	n = 175 1.0±0.102 (0.892-1.102)
Lactase	n = 25 0.581±0.610 (0.0-1.191)	n = 16 0.817±0.262 (0.555-1.080)	n = 44 1.0±0.208 (0.794-1.206)
ALP	n = 25 0.525±0.162 (0.363-0.687)	n = 29 1.051±0.247 (0.803-1.299	n = 132 1.00±0.089 (0.911-1.089)
Residual activity (%)	n = 25 0.668±0.110 (0.558-0.778)	n = 29 1.022±0.049 (0.973-1.071)	n = 128 1.0±0.030 (0.970-1.030)
GGT	n = 25 0.378±0.101 (0.278-0.479)	n = 29 1.059±0.186 (0.878-1.245)	n = 132 1.0±0.079 (0.921-1.079)

(Since lactase activity is very low in the second trimester, and measurement of a reduced value is not reliable, therefore these analyses were dropped).

If a single enzyme activity was greater than or equal to the Xcrit value, it was classified into the "healthy", if it was lower into the "affected" group.

If we include individual enzyme values retrospectively into the "healthy" and "affected" groups, we get the detection rate for each enzyme activity (Table II).

Since it is believed that with the combination of several parameters the health status of the fetus can be evaluated with a greater accuracy, all the parameters, including the Xcrit value have been taken into consideration both in the group of

TABLE II

a) Detection rate for individual enzyme activities

Enzyme /Xcrit value/	Affected fetus found/total ("sensitivity")	Healthy fetus found/total ("specificity")
Trehalase /0.832/	23/25 (92.0 %)	67/125 (53.6 %)
ALP /0.817/	22/25 (88.0 %)	77/125 (61.6 %)
Inhibition (%) /0.808/	18/25 (72.0 %)	113/125 (90.4 %)
GGT /0.621/	24/25 (96.0 %)	97/125 (77.6 %)

b) Reliability of individual enzyme activities

Enzyme	total found/total cases
Trehalase	90/150 (60.0 %)
ALP	99/150 (66.0 %)
Inhibition (%)	131/150 (87.3 %)
GGT	121/150 (30.7 %)

high risk pregnancies for CF and that of the control group (Table III).

From the data in Table III we can conclude:

1) In 92 % of affected fetuses (23 cases) at least three or four enzymes (parameters) are below the Xcrit value.
2) In 92 % of healthy fetuses (115 cases) at least two or more parameters are above the Xcrit value.
3) In 95.55 % of the 2/3 CF-heterozygotic group (28 cases) at least two or more parameters are above the Xcrit value.

According to these criteria, the cases can be classified into four groups:

True positive cases: 23
True negative cases: 143
False positive cases: 11
False negative cases: 2

TABLE III

Patterns of microvillar membrane enzymes in affected, healthy and 2/3 CF-heterozygote groups

Enzymes				Affected	Healthy	2/3 CF-
Tre	ALP	Inhib	GGT	fetus	fetus	heterozygotic
-	-	-	-	13	5	1
-	-	-	+	1	1	0
-	-	+	-	6	4	0
-	+	-	-	1	0	0
+	-	-	-	2	0	0
-	-	+	+	0	13	4
-	+	+	-	1	12	4
+	+	-	-	1	0	0
+	-	+	-	0	5	1
-	+	-	+	0	2	2
+	-	-	+	0	4	0
+	+	+	-	0	2	0
+	+	-	+	0	0	1
-	+	+	+	0	21	5
+	-	+	+	0	16	6
+	+	+	+	0	40	5
Total				25	125	29

(-) = measured value < Xcrit

(+) = measured value > Xcrit

On the basis of the above results, we have concluded that in the case of a combination of the four amniotic fluid parameters, the reliability of the method is 92.73 %, its sensitivity is 92.00 %, and its specificity is 92.86 %.

DISCUSSION

Disaccharidases develop in the gastrointestinal tract of the intrauterine fetus in the 11-23 gestational weeks, primarily in the small intestines. Trehalase production increases significantly in the 10-23 weeks, lactase is synthetized mainly in the last months of pregnancy /6/. The membrane-bound disaccharidases originate from the brush-border cells along the microvilli of the small intestines. The villi appear in the 8-10 weeks of fetal life and undergo a maturing process during gestation. The various disaccharidases located on them enter the amniotic cavity through fetal defecation and show a specific profile.

From the 10th gestational week, their value increases, and they reach their maximum in the 14-17 weeks, which is followed by a sudden decrease, and from the 21st week, their level is very low.

The sudden decrease of disaccharidases is explained by the innervation of the anus spinchter after the 20-21 weeks when fetal defecation ends or by the sudden change in the permeability of the intestinal mucosa, inhibiting enzyme outflow /9/.

In consequence, after the 20-21. weeks, because of the very low enzyme levels, the disaccharidases are not suitable for diagnosis.

We have called attention to the importance of trehalase enzyme analysis in the prenatal diagnosis of CF /14, 15/

The suitability of intestinal alkaline phosphatase isoenzyme for the prenatal diagnosis of CF was first shown by Brock et al /2, 3/. This enzyme prevails in the intestinal form in the amniotic fluid in the second trimester. It is of fetal origin and it enters the amniotic fluid during early defecation

supposedly from the desquamed mucosa cells. Overall ALP activity of the amniotic fluid has a tendency to decrease in the 15-20 weeks of pregnancy, but the distribution of forms inhibited by phenylalanine (L-Phe), intestinal and placental inhibitors or by homoarginin (bones, liver, kidney) is constant. In the case of fetal CF, the enzyme activity of the L-Phe inhibited form decreases significantly.
GGT occurs in high concentration in tissue microvilli. They have a role in the absorbing and secreting processes taking place on the epithelial cells of the brush-border membrane. In CF, GGT activity decreases significantly, which can be explained by developmental disturbances, and secondary atrophization of microvilli, or by the presence of an enzyme inhibitor secreted into the amniotic fluid /1, 5/.

Although several studies have confirmed the suitability of the enzymes in question for CF diagnosis, in the absence of standardized methods, the results cannot be compared. Kleijer et al /7/ considered 10 percentile as the lowest limit. According to them, trehalase and lactase enzymes are less informative. By combining GGT and ALP, Aitken et al /1/ found that the sensitivity of fetal CF detection was 84 % (if the lowest limit was 5 percentile with GGT), or 90 % (if in the presence of 2.5 mmol of Phe, residual ALP activity 80 %), thus the predictability of the affected fetus was 28:1 (96.5 %) /1/ Peretz et al /12/ considered 0.5 MoM and 52 % residual activity as cut off values. On the basis of their evaluation, predictive values of different enzymes for affected states are: ALP 69.4 %, inhibition: 93.2 %, GGT: 68.9 %.

In a multicentric study, by retrospective analysis of 258 cases, Brock concluded that if out of the amniotic fluid enzymes two or three had a lower value than 0.5 MoM in the relevant gestational week, fetal CF had a very good diagnostic predictability in the 17-20 weeks of pregnancy (false positive ratio 2.3 %, false negative ratio 4.4 %) /4/

Since these examinations were performed in different gestational weeks, MoM values had to be used for comparing these cases. The values of healthy normal controls measured in

a given week were related to those of high risk cases, and the groups were discriminated on the basis of the Xcrit values.

Our results show that trehalase, ALP, ALP-isoenzyme and GGT enzyme activities in the amniotic fluid measured in the 16-21 gestational weeks, in spite of their non specificity for CF, have not only a very good predictive value for fetal CF, but they also exclude the possibility of the disease.

Tissue specificity seems to be an important criterion in diagnoses based on amniotic fluid enzyme activity. Intestinal ALP isoenzyme proved to be much more reliable and specific than the less specific trehalase or GGT. Our results confirm this assumption. Though the differences between certain parameters of the group are highly significant, by combining enzyme values, fetal health status can be predicted much better and by this method, the number of false negative and false positive cases can also be reduced . By measuring overall enzyme activities, the reliability of the method is over 92 %. In this case, sensitivity and specificity also come to 92 %.

During the past few years, the use of first trimester DNA analysis for prenatal diagnosis of CF has brought about revolutionary changes. The predictability of CF from chorion villus is as high as 100 %. It has led us to believe that second trimester amniotic fluid enzyme assay with 92 % reliability should be used for the prenatal diagnosis of CF in the following cases:

(1) if the CF-heterozygotic couple, or the affected child are informative for none of the available RFLP (restriction fragment length polymorphism) markers:

(2) if the CF-heterozygotic couple has no living affected child, or the DNS of the stillborn affected child is not available:

(3) if, due to advanced gestational age, there is no possibility of genotypifying family members:

(4) if in the 18-20 gestational weeks, routine ultrasound examination is suggestive of meconium ileus in an otherwise not high-risk pregnancy on the basis of its medical history.

Since, even in 1988, more than 50 % of the CF-heterozygotic couples belonged to one of these four groups at our Genetic Counselling Center, mid-trimester amniotic fluid enzyme diagnosis is considered to be a valuable diagnostic method and its use is advisable in the prenatal diagnosis of CF.

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