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Improved standards for prenatal diagnosis of citrullinemia

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ABSTRACT

Citrullinemia type I is a urea cycle disorder caused by autosomal recessive mutations in argininosuccinate synthetase 1 (*ASS1*). In the classical form of this disease, symptoms manifest during the neonatal period as progressive lethargy, poor feeding, and central nervous system depression secondary to hyperammonemia. In pregnancies involving two carrier parents, prenatal diagnosis is important for both reproductive decisions and advanced preparation for neonatal care. The current gold standard for prenatal diagnosis has been the citrulline incorporation assay in addition to DNA mutation analysis. Herein, we review our experience with prenatal diagnosis of citrullinemia type I over the span of 11 years in 41 at-risk pregnancies. During this time, we identified 15 affected fetuses using a combination of molecular and biochemical testing. Given the established limitations of both the citrulline incorporation assay as well DNA mutation analysis, we probed our data to assess the value of amniotic fluid amino acid levels in prenatal diagnosis. Previous publications have proposed using the amniotic fluid ratio of citrulline/(arginine + ornithine) in prenatal diagnosis; however, we noted that amniotic fluid arginine levels were normal in our cohort and hypothesized that the amniotic fluid citrulline/ornithine ratio may be superior. Indeed, our analyses revealed that the ratio of amniotic fluid citrulline/ornithine alone correctly distinguished affected from unaffected fetuses in all cases. During the establishment of a normal reference range we discovered significant elevations in amniotic fluid citrulline levels in at-risk pregnancies compared to the normal population even when the fetus was unaffected. This highlights the importance of using amniotic fluid from carrier mothers when setting up a normal reference range. Finally, we report our experience as one of the first centers to adopt Sanger sequencing for prospective prenatal diagnosis of citrullinemia. While this is clearly a useful tool in many cases, we encountered families for whom molecular analysis uncovered variants of unknown clinical significance or no mutation at all. Based upon these new findings, we recommend a combinatorial approach involving *ASS1* sequencing and amniotic fluid citrulline/ornithine for the prenatal diagnosis of citrullinemia type I.

1. Introduction

Citrullinemia type I (MIM 215700) is an autosomal recessive urea cycle disorder caused by deficiency of the enzyme argininosuccinate synthetase (AS) [1]. AS plays a key role in the removal of waste nitrogen by catalyzing the condensation of citrulline and aspartate to form argininosuccinate. Thus patients with AS deficiency typically have marked baseline increases in plasma citrulline punctuated by periods of hyperammonemia. Clinical features of the classical form of citrullinemia typically present in the neonatal period with progressive lethargy, tachypnea, and poor feeding secondary to hyperammonemia. If left untreated, patients can experience irreversible neurological injury, coma, and death. Milder late-onset forms of citrullinemia have been described and can be distinguished from the classic neonatal form on the basis of plasma citrulline levels, *ASS1* allelic findings, and the timing/severity of symptoms [2–4].

In high-risk pregnancies involving two carrier parents, prenatal diagnosis of classic citrullinemia is critical for reproductive planning and neonatal management. A variety of biochemical methods have been employed in the prenatal diagnosis of citrullinemia, including assays of AS enzyme activity and measurement of amino acid levels in amniotic fluid [5–7]. Enzymatic approaches are completed using either amniotic or chorionic villus cells and can be divided into two categories: (i) direct testing of AS enzymatic activity [8] and (ii) indirect assay of AS activity via the measurement of ^{14}C-citrulline incorporation into polypeptides [7,9]. Additional diagnostic information is gained via the measurement of amino acid levels in amniotic fluid, with most affected fetuses exhibiting elevations of amniotic fluid citrulline (AF-Cit) clearly above those found in normal pregnancies [5–7]. In pregnancies with borderline AF-Cit values, improved diagnosis has been proposed using amniotic fluid ornithine (AF-Orn) and arginine (AF-Arg) to form a

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Abbreviations

AF-Cit	amniotic fluid citrulline
AF-Arg	amniotic fluid arginine
AF-Orn	amniotic fluid ornithine
ASS1	argininosuccinate synthetase 1
VUS	variant of unknown clinical significance

ratio with citrulline, [AF-Cit]/([AF-Arg] + [AF-Orn]),instead of AF-Cit alone [6].

To date, reports of molecular analysis for prospective prenatal diagnosis of citrullinemia have been limited to *ASS1* intragenic dinucleotide repeat linkage analysis but such studies are prone to equivocal results [5,10]. As an alternative, direct sequence analysis of the *ASS1* coding region has been reported to identify mutations or potential causative variants in over 90% of classic citrullinemia cases; however, the detection rates of variants of unknown clinical significance are high and the diagnostic yield in multi-ethnic populations receiving prospective prenatal testing remains unclear [7,11].

The following report describes our laboratory's experience with the prenatal diagnosis of citrullinemia using a combination of testing strategies. Analysis of our clinical data allowed for the optimization of our detection rates using amniotic fluid amino acid ratios as biomarkers for disease. We also report the first long-term clinical application of *ASS1* sequencing for the prenatal diagnosis of citrullinemia. Taken together, our data suggest *ASS1* sequencing combined with biochemical testing, especially of amniotic amino acids, should be the new gold standard in the prenatal diagnosis of citrullinemia.

2. Material and methods

2.1. Amniotic fluid citrulline measurement

Amniotic fluid was stored at −20 °C for no more than 7 days prior to testing. For the majority of pregnancies studied, the gestational age at time of amniotic fluid sampling was between 15 and 18 weeks. "Low-risk unaffected" samples were collected from pregnancies receiving testing for concerns related to aneuploidies, such as advanced maternal age or abnormal maternal serum screen results. For our study, we included only those specimens that were subsequently found to have a normal karyotype. Sample preparation involved protein precipitation and sample protonization using equal volumes of seraprep (Pickering Laboratories) and 200 mM lithium citrate (pH = 2.2). Filtered supernatant was analyzed via cation exchange chromatography using either a Biochrom 30 or Hitachi L-8900 amino acid analyzer. p-Values were calculated using a 1-tailed heteroscedastic student's t-test. In cases of equivocal prenatal results, confirmation of disease status was achieved through postnatal follow-up.

2.2. AS enzyme analysis

AS enzyme analyses were completed on cultured amniocytes or chorionic villus as described previously [5]. Briefly, cells were grown to confluency in minimal essential media (MEM), replated at 10^5 per 60 mm dish, and grown overnight. The following day the media was replaced with -Leu and -Arg MEM supplemented with ^{14}C-citrulline and ^{3}H-leucine, and cells were grown for an additional 6 h prior to harvesting. In the presence of normal AS and argininosuccinic acid lyase (ASL) activity, ^{14}C-citrulline was converted into ^{14}C-arginine. The incorporation of ^{14}C-arginine within polypeptides was used to determine AS and/or ASL activity. As a control for general polypeptide production and cell growth, ^{3}H-leucine was also measured.

2.3. Molecular analysis

Fetal DNA was extracted from chorionic villus or amniocytes using a Puregene DNA Purification kit according to the manufacturer's instructions (Qiagen). Linkage analysis was performed for samples received prior to May, 2006 as previously described (7). For *ASS1* sequence analysis, the proband's DNA was first analyzed by sequencing the coding exons and adjacent flanking intron regions, as previously described [3]. Parental carrier status was confirmed in most of the cases, and fetal samples were evaluated only for the familial mutations through targeted *ASS1* sequence analysis.

3. Results/discussion

Over the last 11 years, our laboratory has completed prenatal testing on 41 pregnancies at high risk for citrullinemia (Table 1). To qualify for testing, families were required to have at least one prior child diagnosed with the classical neonatal form of citrullinemia. This was an important measure to ensure our findings were not confused by hypomorphic or benign variants. Ultimately, 15 cases were determined to be affected. Diagnosis was achieved in part using a variety of biochemical approaches, including citrulline incorporation assay alone (n = 12), concentration of AF-Cit alone (n = 1), or a combination of both (n = 28). Many cases also had molecular analysis using either linkage (n = 12) or Sanger sequence analysis of exons 3–16 of *ASS1* (n = 14) (Table 1). For 40 of 41 pregnancies, we reported a definitive diagnosis. In the one case we did not reach a conclusion, biochemical results were borderline, and molecular testing was not ordered; the fetus was ultimately born unaffected (patient #17; Table 1).

Nearly all pregnancies tested (40 of 41) received citrulline incorporation studies on cultured amniocytes (28) or chorionic villus (12). In our experience, the starting material impacted the quality of the test. For assays completed on cultured chorionic villus, there was a clear delineation between normals and affected in all cases with a confirmed diagnosis. However, citrulline incorporation values were highly variable for normal specimen raising some concern about the precision of this test (2.5–56.9 ^{14}C/^{3}H) (Fig. 1A and Table 1). Assays performed on cultured amniocytes fared worse. Five patients had borderline values that could not yield a definitive diagnosis without further data (patients #5, 8, 17, 20, and 23; Fig. 1B and Table 1). The variability observed in citrulline incorporation assay results may be explained in part by differences in cell culturing efficacy or inherent biological variation. Importantly, enzymatic values were in concordance with AF-Cit values and in some instances provided additional support when AF-Cit values were ambiguous (Fig. 1B).

For the majority of pregnancies, AF-Cit levels alone were a robust predictor of affected status. To improve the diagnosis in cases of equivocal values, a ratio of [AF-Cit]/([AF-Arg] + [AF-Orn]) had previously been proposed but was found to have limited utility in some cases [6,7]. To test the possibility of using a ratio in our dataset, we first compared overall amniotic fluid amino acid profiles between the affected (n = 11) and unaffected (n = 18) populations. As expected, based on the position of AS in the urea cycle, AF-Cit levels in affected pregnancies were significantly elevated ($p = 1.18 \times 10^{-6}$), and ornithine levels (AF-Orn) were significantly reduced (p = 0.02). Surprisingly, arginine (AF-Arg) was not significantly reduced (Fig. 2). We therefore hypothesized that the diagnostic accuracy of amniotic fluid amino acids could be improved by calculating AF-Cit/AF-Orn and applied this ratio to our dataset to compare its utility on a case-by-case basis. Using AF-Cit alone, four cases were equivocal with values between 30 and 50 μM (patients #3, 16, 17, 32; Table 1) (Fig. 3A). Improved separation was achieved between affected and unaffected patient populations in all cases when employing a ratio, either [AF-Cit]/([AF-Arg] + [AF-Orn]) or [AF-Cit]/[AF-Orn] (Figs. 3B and C). However, only the optimized ratio of [AF-Cit]/[AF-Orn] provided the correct diagnosis in every case. The original ratio of [AF-Cit]/([AF-Arg] + [AF-Orn]) failed to provide

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