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CASE REPORTS

Preserved Blood Spots Aid Antenatal Diagnosis of Citrullinemia Type-1

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Abstract Inborn errors of metabolism are an important cause of non-communicable under-five childhood mortality. Lack of confirmatory 'genomic' results in the deceased index case due to unavailability of post-mortem biological samples, can pose challenges in reproductive counseling of the parents in future pregnancies. Our case describes a couple seeking preconception genetic counseling after they lost their previous child to biochemically diagnosed Citrullinemia type-1. We confirmed the genomic diagnosis of Citrullinemia type-1 through the post-mortem genetic analysis of the DNA retrieved from the preserved blood spots, 12-months later. Prenatal testing in the next pregnancy revealed the fetus to be a carrier for Citrullinemia type-1. This case report intends to raise the obstetricians' and neonatologists' awareness regarding DNA banking in fatal genetic disorders and the mandatory confirmatory genetic diagnosis for effective prenatal genetic counseling.

Keywords Citrullinemia · Prenatal diagnosis · Genetic counseling · DNA banking

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Background

Inborn errors of metabolism (IEOM) are a heterogeneous group of inherited defects in metabolic pathways, resulting in a varied spectrum of clinical features including neurodevelopmental abnormalities, encephalopathy, seizures, liver dysfunction, cardiomyopathy; sometimes serious morbidity, even premature death. Inability to provide a precise genetic diagnosis while the index child is still alive can pose challenges in counseling regarding the recurrence of the same condition in future pregnancies [1, 2].

Case Report

A 24y old woman, married to her first cousin (third-degree consanguinity), presented to us for preconception genetic counseling. She had delivered a healthy male a year ago at full-term after an uneventful antenatal period. The baby's APGAR score was 9/10 and birth weight 3100 g. Breastfeeding was initiated on the first day. The mother recalled good sucking and vigorous activity, until the second day, when the child developed refusal to feed and progressively increasing drowsiness. Over the next two days, he developed multiple episodes of seizures, deepening coma and succumbed on day 6. Biochemical tests carried out on day 5 pointed towards the possibility of Citrullinemia type-1 (Table 1).

The couple was aware of a 25% chance of recurrence of the same disorder in their subsequent pregnancies. We discussed the mandatory need for identifying the pathogenic disease-causing mutation in the proband through genetic tests to provide a definitive prenatal diagnosis (PND) in the future. The family faced the dilemma of not having any banked biological sample of the baby for the proposed genetic tests. Our plan B, targeted Sanger sequencing of the gene responsible for Citrullinemia type-1 (i.e.*ASS1*) in the couple, to detect their carrier status, was declined because of cost considerations. They were also counseled about the lower diagnostic yield of indirect parental testing versus the proband-first approach because de novo mutations that occurred in the proband would be missed. Fortunately, the primary laboratory, which had done the biochemical tests confirming Citrullinemia, had not discarded the dried-blood-spot (DBS) card of the deceased baby, even after 12 months.

DNA was retrieved from these DBS cards using QIAamp DNA Mini Kit and subjected to targeted Sanger sequencing of *ASS1 gene* using the Big Dye Terminator Cycle Sequencing kit version 1.1 and an ABI 3130 genetic analyzer (Applied Biosystems by Life Technologies Europe BV, Zug, Switzerland). The test was conducted through research grants at the Zurich laboratory. The proband was detected to harbor a known missense variant c.1168G > A (p.Gly390Arg) in exon 15 of *ASS1* gene in homozygous state (Fig. 1). At a molecular level, the mutation was classified as ACMG class-5, definitely pathogenic, resulting in complete inactivation of argininosuccinate-synthetase enzyme activity [3]. This mutation is globally present and is the single most frequent variant in the *ASS1* gene. It is already described in several patients (most of them homozygous carriers) with neonatal onset severe citrullinemia [4]. Both parents were found to be obligate heterozygotes (carriers) for the same variant (Fig. 1). Thus, a confirmed genetic diagnosis of Citrullinemia type-1 was now established in the index patient.

Two months later the couple conceived spontaneously. Since the genotype in the proband was proven to be severe, PND by molecular genetic means could be offered to the couple. Chorion villus sampling (CVS) was performed at 12 weeks of gestation (Fig. 2). Purified fetal DNA, after ruling out maternal-cell-contamination, was subjected to targeted Sanger analysis for the above family-specific mutation (c.1168G > A in *ASS1*). It revealed the fetus to be a carrier for the given condition. The couple delivered a healthy male child at full-term.

Discussion

IEOM, a heterogeneous group of over 700 conditions, although individually rare, are collectively common [1]. Amongst these, Citrullinemia type-1 is a type of urea cycle disorder (UCD) [2, 5]. In a recent series of 123 children with UCD, Citrullinemia type-1 has been estimated to be the commonest UCD in India [2].

Table 1 Summary of the available investigations

Name of test	Result	Interpretation
1. Ratio Urinary Orotic acid/ Creatinine	815 µmol/mmol Creatinine (normal range 0–30)	Suspicion of UCD [a]
2. Plasma carnitine and acylcarnitine levels	Normal	
3. Plasma Amino Acids (TMS[b] on DBS[c])		
a. Alanine	5008.38 µmol/L (Cut-off 600)	Suggestive of UCD, especially Citrullinemia type-1
b. Glycine	1969 µmol/L (Cut-off 1000)	
c. Citrulline	3975.08 µmol/L (Cut-off 55)	
d. Glutamic acid	3928.94 µmol/L (Cut-off 1070)	
c. Other AA	Normal	
4. Plasma amino acids (UHPLC)[d]		
a. Citrulline	4061 µmol/L (normal range 19–52)	Biochemical confirmation of Citrullinemia type-1
b. Glutamine	2931 µmol/L (normal range 457–857)	
c. Other AA	Normal	
5. Urinary GCMS[e]	Normal	

[a]: UCD: urea cycle disorder[b]: TMS-Tandem mass spectroscopy,[c]: DBS-dried blood spots, [d]: U-HPLC-ultra high performance liquid chromatography, [e]: GCMS-gas chromatography mass spectroscopy

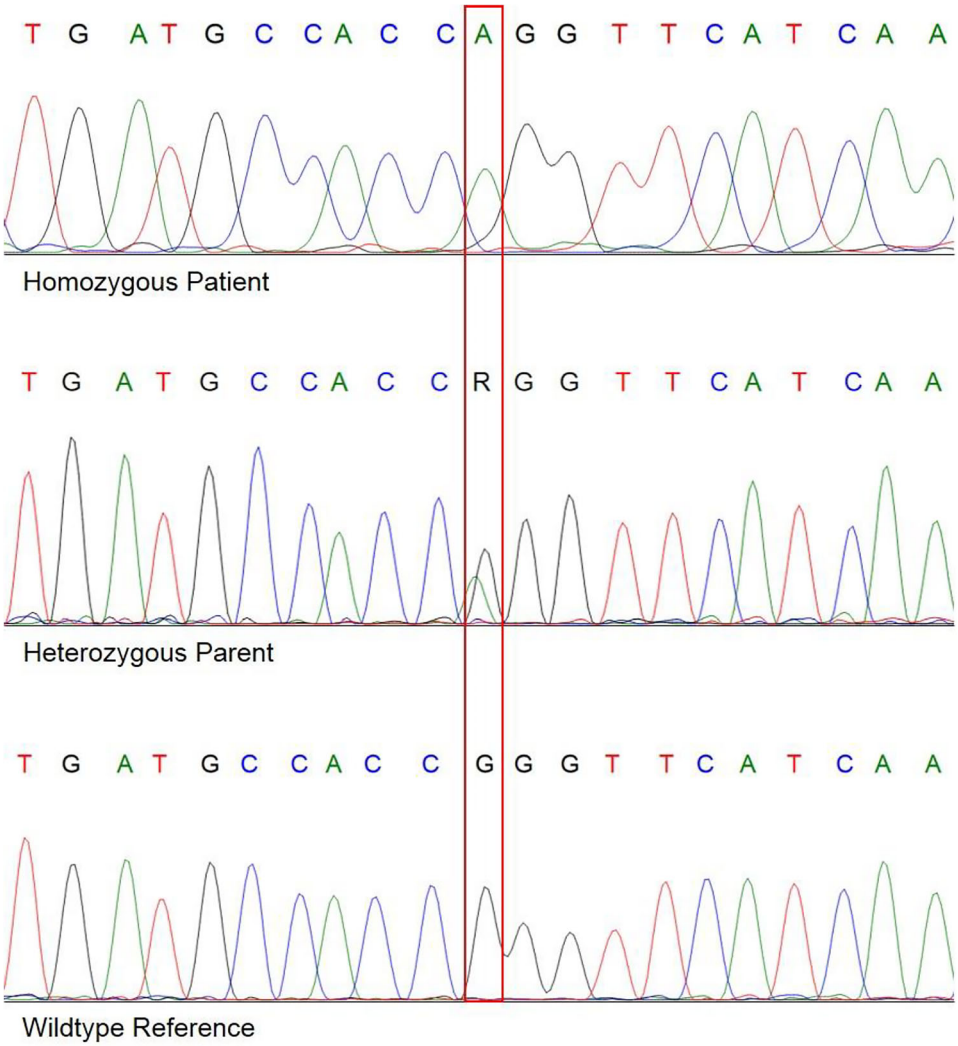

Fig. 1 Sanger sequencing image of *ASS1* missense mutation c.1168G > A, p.Gly390Arg

Why Would Parents Usually Demand Prenatal Diagnosis (PND) in Citrullinemia?

Mortality due to UCD is usually high (88%), almost 90% occurring in the neonatal period [2]. Although treatment options are now available even in India, there exist challenges to prevent the serious neuro-morbidity and mortality associated with UCD, even in children who receive treatment [2]. Citrullinemia type-1 follows an autosomal-recessive trait, carrying a 25% risk of recurrence in future pregnancies [2, 5]. Previous sibling affection and death were reported in one-third of the families in the above series by Bijarnia-Mahay et al. [2]. Besides the emotional burden of child loss, the cost of biochemical and genetic tests, special dietary formulae, repeated hospital admissions, regular laboratory surveillance and management of the acute decompensations in UCD, involves an additional, often overlooked, economic burden to these families [6]. Thus, most couples with a history of child loss due to UCD, are keen to prevent the same in the future, by the appropriate utilization of PND or preimplantation-genetic-diagnosis (PGD) [2, 5]. However, clinical or biochemical diagnosis, even if confirmatory are inadequate for PND or PGD; a precise genetic diagnosis being quintessential in such scenarios [2, 5].

DNA Extraction from Preserved Samples

There exist multiple challenges for the lack of confirmatory genetic testing in the index case, while it is still alive. Few relevant ones are lack of physician awareness about the evolving spectrum of newer genetic tests beyond the "good-old" karyotype, prohibitive costs of these sophisticated DNA-based tests and early death in serious and fatal genetic disorders [2, 7, 8]. The hunt for a biological sample of the index case, months to years after its death, can be challenging. In the absence of the proband's DNA sample, indirect parental testing can sometimes be an alternative, as

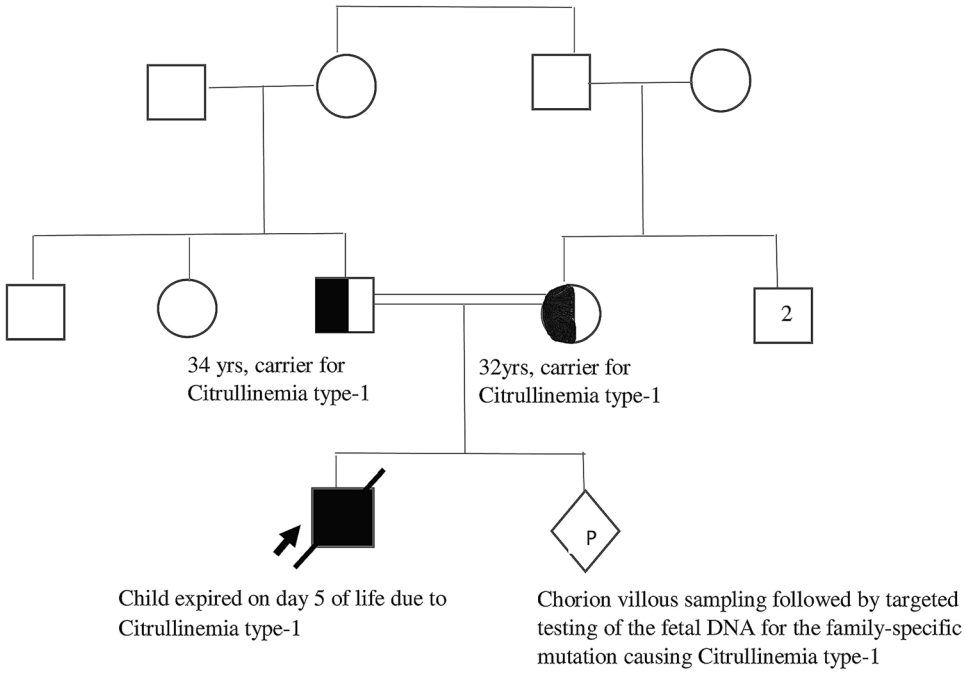

Fig. 2 Pedigree chart of the family seeking genetic counseling

was offered in our case. However, the pitfalls of this approach caution one to opt for it only when there is absolutely no means of obtaining the proband's sample [9]. For instance, if the parental testing yields a variant of uncertain significance (VUS), the relevance of the same would ideally need to be assessed in the proband's sample [9, 10]. As per ACMG guidelines, PND in future pregnancies, based on such VUS, is discouraged, making the exercise sometimes futile and the results, unactionable [10]. Further, a negative carrier testing would reduce the risk, but not negate the same for any suspected autosomal or X-linked recessive disorder. Thus, the importance of pursuing the 'proband first' test approach, whenever possible [9]. There are reports of post-mortem genetic diagnoses of the proband using shriveled umbilical stumps of the child [11, 12] and even a single strand of hair [13]. India is looking positively at the prospect of 'universal' NBS using DBS cards, for select disorders in the near future [14]. This report highlights an already known potential of preserving these DBS cards for future genetic testing.

DBS is a reliable source of DNA for targeted gene analysis by Sanger sequencing and can even be used for NGS-based analysis, keeping in mind the consideration of fluctuating coverage and only partial In-Del detection. [15] However, DNA from DBS is not recommended for MLPA analysis due to the low amount of DNA usually obtained from DBS. [15, 16] In screen-positive cases of NBS, it is likely that the DBS spots could sometimes be re-utilized to verify the results and hence sufficient DNA may not be available for further genomic studies in the future [15]. For best results, it is recommended that the DBS spots be appropriately collected and standardized in the concerned laboratory [15, 16]. In the absence of NBS, simple and inexpensive DNA banking facilities, using the proband's blood sample (2-ml, EDTA-vacutainers), are now available on research as well as a commercial basis (range INR 500-3500), depending on the laboratory performing the DNA extraction and the duration for which the sample is stored (range 6–42 months) [personal communication]. DBS cards can be preserved for up to 10–15 years at + 2 to + 8 degrees Celsius. [17] Certain research laboratories, like the center in our study, preserve the DBS cards and other diagnostic samples for as long as ten years or more, sometimes at no additional cost (Fig. 3). CVS samples, amniotic fluid, skin biopsy from stillbirths, and even products-of-conceptus can be utilized for banking the DNA of the fetus [11]. Tapping on the under-utilized potential of banked DNA for post-mortem genetic analysis to provide answers in preconception and prenatal genetic counseling scenarios should be reflexive and an easily accessible option, rather than a result of serendipity, as in our case and other reports.

With rapid expanses in our understanding of genomics and its role in diseases, the practicing obstetrician-gynecologist will be increasingly expected to incorporate these principles in day-to-day prenatal care and counseling [7]. While the case highlights the strength and potential of modern genomics, it cannot be overemphasized that the success of these sophisticated tests depends on simple and robust first-line and ancillary tests, exemplified by a detailed biochemical work-up in this case. Review of the

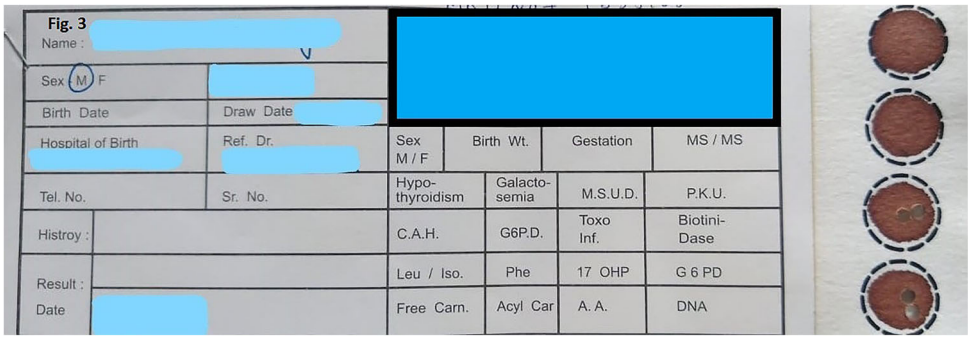

Fig. 3 An example of a well-prepared dried blood spot (DBS) card

family history, detecting the red flags for genetic disorders, discussing the option of DNA storage with families in suspected genetic disorders, heightened awareness about the advances in molecular genetics and its applications, and synchronized team-efforts with allied clinical branches, together, can go a long way in guiding families with genetic diseases in a holistic manner [7].

Implication for Clinical Practice

Banked DNA and its application for appropriate genetic testing can help in effective prenatal and preconception genetic counseling, even after the death of the index case.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SB and UJ. The first draft of the manuscript was written by SB and all authors commented on the different versions of the manuscript. All authors read and approved the final manuscript. AJ laboratory had preserved the blood spots for further analysis. JH and VR were involved in the research-based molecular genetic testing of the patient and the parents. SB will act as the guarantor of the paper.

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Declarations

Conflict of interest None.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki.

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