

The Utility and Futility of Targeted Next Generation Sequencing for Carrier Detection in 'At Risk' Couples

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Abstract

Next generation sequencing has changed the approach to genetic diagnosis and testing in recent times. The days have arrived when a molecular genetic diagnosis can be attempted even without the availability of the proband or affected person. However this requires additional strong evidence of diagnosis in the proband, such as biochemical or radiological hallmarks. Needless to say, this attempt should always be made during counseling to make the family aware of the fallacies and limitation involved due to non-availability of the sample of the proband. Since many recessive disorders are either fatal or severely debilitating and burdensome, with no easy treatment, the NGS technology has provided a much needed relief in terms of testing and prevention in family by enabling prenatal diagnosis, at least in a few cases. Encouraging results have been shown upon testing of the proband directly. However, the final word remains to be said on complete reliability of the method for carrier testing, performed without availability of the proband. We present two similar cases, of methyl malonic acidemia, where use of NGS resulted in contrasting outcomes after genetic testing for carrier status, thus highlighting the utility and the futility of the test in certain situations. While a correct identification of mutations in the first couple (in the *MMAB* gene) resulted in successful prenatal diagnosis and prevention of recurrence, an inability to identify the disease and

mutation resulted in birth of an affected baby with MMA in the second family.

Introduction

Next generation sequencing (NGS) technology has revolutionized genetic testing by providing an easy, and relatively quick and accurate solution to the 'undiagnosed genetic disorders' group (Katsanis et al., 2013). It has landed in a big way in India, especially for enabling prenatal diagnosis, since majority of families, at least in our set up, present with the hope of prevention of recurrence of their burdensome disease, in their ongoing or future pregnancies (Puri et al., 2016). Therefore, the main purpose of genetic testing and diagnosis in the proband or family member is essentially to be able to make a confirmed molecular diagnosis, so as to enable an accurate prenatal diagnosis. NGS in parents of an affected child, where the index case or proband is unavailable, is not that straightforward. In specific situations where a phenotype is clearly defined, and there is an additional strong evidence available (such as biochemical or radiological), carrier testing may be considered. Inborn errors of metabolism, where the diagnosis is usually achieved using biochemical metabolic testing, is one good example. However, there remain a lot of uncertainties if this approach is applied, as illustrated by the two cases described here. The two cases chosen had a similar background history and ultimately proved to have the same diagnosis,

but with vastly different consequences after using the same technology. Both cases had a diagnosis of methylmalonic acidemia, which is the commonest organic acidemia in India (Verma et al., 2005). Methylmalonic acidemia is an autosomal recessive IEM of energy production. The clinical presentation is very variable, from neonatal fatal presentation if unrecognized, to mild intermittent illness. The disorder is manageable if recognized in time, and appropriate treatment is instituted (Baumgartner et al., 2014).

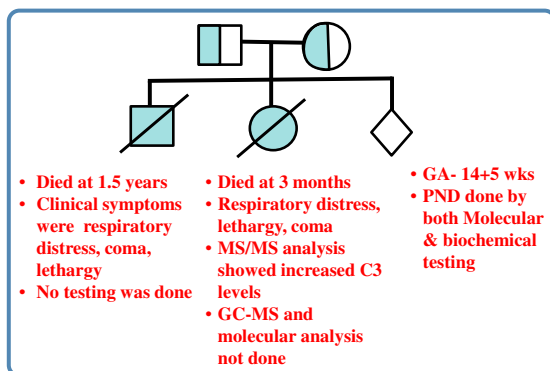


Figure 1 Case 1: Pedigree with clinical details of the two affected children.

Case 1

A non-consanguineous couple, from Goa, presented to us in the pre-pregnancy planning stage for genetic counseling. The family had lost two children at 1.5 years and 3 months of age, with episodes of respiratory distress, lethargy and coma with no clear diagnosis (Figure 1). Tandem mass spectrometry (MS/MS) analysis in the second baby had shown increased C3 metabolite, suggesting the possibility of organic acidemia. Due to non-availability of the proband's DNA, the couple was tested, sequentially. The wife's DNA was tested for multigene panel of organic acidemias using NGS. A heterozygous novel mutation, c.2T>G, in the initiation codon, was detected in the *MMAB* gene. In-silico analysis predicted pathogenicity. The variation, c.2T>G was considered pathogenic as the initiation codon is lost. The highly conserved 'T' of ATG is substituted by 'G'. Due to this change, translational process does not start at the expected position and requires an alternative downstream initiation codon (Wolf et al., 2011). The next in-frame methionine codon is present 600bp downstream and would comprise approximately 50 amino acids. If it is used it is expected to

lead to a truncated protein without the N-terminal domain and hence is unlikely to be functional. The MutationTaster software predicted this mutation to be disease-causing.

After validating the mutation using Sanger sequencing, the husband's DNA was sequenced and noted to carry the same mutation (Figure 2). Prenatal diagnosis was performed in the subsequent pregnancy, using amniotic fluid for metabolite testing through Gas Chromatography Mass Spectrometry (GC-MS) as well as targeted mutation analysis. The biochemical analysis on AF showed that the fetus was affected, with elevation of methylmalonate in the amniotic fluid. The same was also confirmed by mutation analysis (homozygous c.2T>G) (Figure 2). The pregnancy was terminated after counselling.

Targeted panel sequencing thus proved useful in this family as it paved the way for prenatal diagnosis and prevention of this burdensome disorder in the family. The results were convincing as there was additional biochemical evidence of disease in the fetus.

Case 2

A non-consanguineous couple from North India presented in the pre-pregnancy period for genetic counseling. The couple had lost a previous baby in the neonatal period. The baby girl was born at term, uneventfully. She was admitted on day 7 of life in NICU with history of lethargy and poor feeding. Baby was in shock at the time of admission, with tachypnoea and severe metabolic acidosis. She was treated on the lines of late onset sepsis, but went into refractory shock on day 2 of admission and succumbed. Routine hematology, biochemistry, liver and renal functions were normal with only mild derangement in the coagulation profile and no evidence of sepsis. Metabolic test (tandem mass spectrometry, TMS) was done which showed elevated propionylcarnitine (C3) metabolite on dried blood spot (11 $\mu\text{mol/L}$, normal <6). There was also increase in a few amino acids (leucine, ornithine, phenylalanine, tyrosine and valine) which suggested secondary metabolic derangement owing to sick state and liver dysfunction. Baby died on day 10 of life, 48 hours after admission.

Considering the presentation of the baby, a heightened suspicion of an organic acidemia was made (severe metabolic acidosis, raised C3 on TMS, no evidence of sepsis). The mother of the

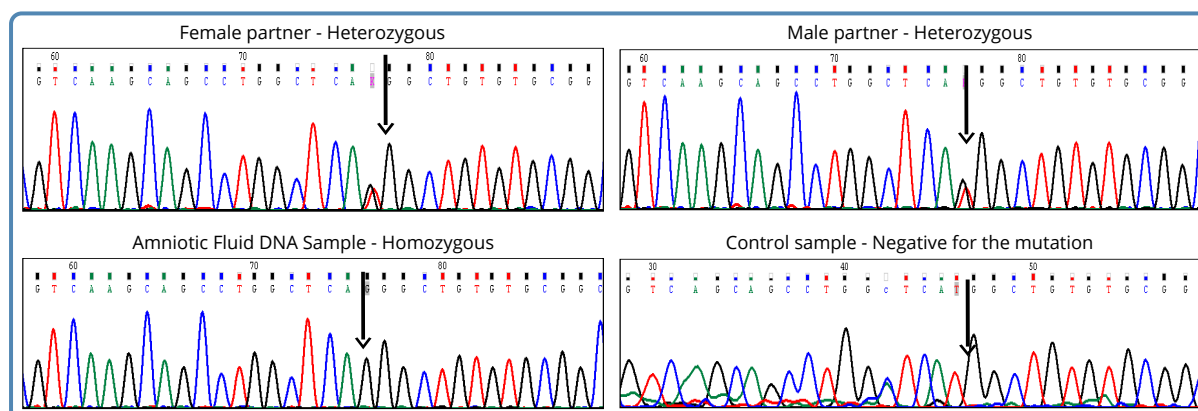


Figure 2 Molecular studies in family (Case 1) showing presence of mutation, c.2T>G in the *MMAB* gene in heterozygous state in the couple, and homozygous state in the fetus (amniocyte DNA), and normal sequence in a control sample.

baby was tested for carrier status, using the targeted organic acidemia panel by next generation sequencing. A panel of genes causing organic acidemia was tested. No specific mutation, correlating with the disease in the baby was noted. However, she was noted to be heterozygous for a novel, possibly significant variant (c.278InsA in *ETFB* gene). Homozygous or compound heterozygous mutations in the *ETFB* gene result in Multiple Acyl CoA dehydrogenase (MADD) deficiency, also known as Glutaric Aciduria type 2. The neonatal form of MADD is usually fatal and characterized by severe nonketotic hypoglycemia, metabolic acidosis, multisystem involvement, and excretion of large amounts of fatty acid- and amino acid-derived metabolites. In view of a positive clinical correlation, this mutation was considered significant and led to testing of the husband. Again, NGS for organic acidemia genes was performed and he was noted to be negative for the same mutation and also negative for any other pathogenic variation in the *ETFB* gene.

The couple was then counseled regarding the non-feasibility of prenatal diagnosis in their subsequent pregnancy owing to failure of detection of their carrier status using the NGS test. The couple went ahead with pregnancy, with routine monitoring and delivered a healthy boy at term, weighing 2.5 kgs. He was kept under observation for 4 days in the hospital and discharged on breast feeds. Newborn screening was performed on day 3 of life using TMS which detected elevated levels of C3 (5.09 nmol/ml, normal <3.5), with normal levels of amino acids. Further testing showed elevated methylmalonic acid (24.2 μ M;

ref.<1) on DBS. Urine GC-MS analysis revealed elevation of methylmalonate, 3-hydroxypropionate and methylcitrate, consistent with the diagnosis of Methyl malonic acidemia. Serum homocysteine level was not elevated. The baby was started on the treatment regimen: Inj. Hydroxocobalamin 1 mg per day intramuscularly, oral carnitine and vitamin supplements and continued on breast feeds. A repeat GC-MS after few weeks of vitamin B12 therapy revealed a marked reduction in the peak of methylmalonate in the urine sample, suggesting B12-responsiveness. The baby is now 16 months old and doing well, gaining milestones appropriately, with no major decompensations requiring hospitalization.

Molecular testing in the baby, using the NGS-based organic acidemia panel did not reveal any pathogenic variation. However, note was made of one large contiguous region corresponding to exon 5 in the *MMAA* gene (chr4:146572210-146572303) that was not covered in the sequence data of the sample, which is usually covered by the test. This suggested possibility of a homozygous deletion of exon 5 in the *MMAA* gene. The same was then tested using PCR using specific primers for exon 5 of the *MMAA* gene (Figure 3), which confirmed the diagnosis of MMA in the child.

Homozygous deletion of exon 5 in the proband was confirmed by multiplexing exon 5 of *MMAA* gene with another unrelated fragment (exon 7 of *HEXA* gene), where exon 7 *HEXA* served as a control fragment. In this multiplex PCR the proband's DNA amplified for exon 7 *HEXA* while exon 5 of *MMAB* gene did not amplify, proving deletion of exon 5. The heterozygous deletion in parents could not be

confirmed by the same PCR as their DNA amplified for exon 5 of the *MMAA* gene.

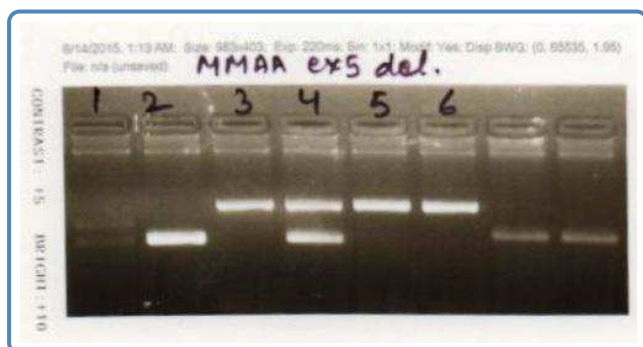


Figure 3 Molecular studies for detection of homozygous deletion of exon 5 in *MMAA* gene in the proband. PCR analysis was performed using specific primers for exon 5 (Lanes 1 & 2) and also multiplexing with another PCR for amplification of a fragment of *HEXA* gene (Lanes 3 & 4) in patient and control DNA. PCR analysis of only *HEXA* gene fragment is also shown (Lanes 5 & 6). Lanes 1, 3 and 5 contain patient's DNA and Lanes 2, 4 & 6 contain control DNA. Lane 1 with patient's DNA shows no band, thus no amplification of exon 5 after PCR using exon 5 specific primers; Lane 2 shows positive band of exon 5 in a control sample; Lane 3 shows positive band for the *HEXA* fragment and no band for the exon 5 *MMAA* gene fragment in patient's DNA; Lane 4 shows presence of both *HEXA* and *MMAA* fragments in control DNA; Lane 5 & 6 show positive bands for *HEXA* in the patient's DNA and control DNA respectively.

Discussion

Methyl Malonic Aciduria (MMA) is a common organic aciduria observed in the Indian population (Verma et al., 2005). Although diagnosis of MMA is achieved by biochemical testing based on abnormal metabolites on MS/MS on dried blood spots and GC/MS in patient's urine sample, molecular diagnosis is still required to provide confirmation of the exact type of MMA and to enable an accurate prenatal diagnosis for subsequent pregnancies. Next generation sequencing is a powerful technology benefitting particularly the average Indian family who wish to undergo prenatal diagnosis to prevent the burden of a genetic disorder in the family. While the technology has been used very successfully in making an accurate genetic diagnosis in probands (Reid; 2016), this has not

been evaluated in the 'at risk' family members who may be carriers. Various levels of testing are now possible using NGS varying from a targeted panel of genes, to whole exome or whole genome sequencing (Katsanis et al., 2013). We utilized the targeted panel of genes to identify carrier status in two couples presenting with a similar history. After going through the similar process of carrier testing, the outcome was starkly different. While next generation sequencing (NGS) enabled prenatal diagnosis by molecular analysis in the first family, with successful prevention of recurrence, this was not possible in the second family. Thus, clinicians should be careful in counseling the families, especially couples who approach for testing, especially in the absence of a proband, about the lack of complete reliability on the NGS for genetic testing.

The two cases also illustrate the finding which is increasingly being noted in other Indian studies, which is the presence of homozygosity even in absence of any consanguinity (Gupta et al., 2016). This is very depictive of the unique system of marriages in our country where small pockets of homogeneity are created within the heterogeneous population, because of a caste or community barrier.

References

1. Baumgartner MR, et al. Proposed guidelines for the diagnosis and management of methylmalonic and propionic acidemia. *Orphanet J Rare Dis* 2014; 9:130.
2. Gupta D, et al. Seventeen novel mutations in PCCA and PCCB Genes in Indian propionic acidemia patients, and their outcomes. *Genet Test Mol Biomarkers* 2016; 20: 373-382.
3. Katsanis SH and Katsanis N. Molecular genetic testing and the future of clinical genomics. *Nat Rev Genet* 2013; 14: 415-426.
4. Puri RD, et al. Genetic approach to diagnosis of intellectual disability. *Indian J Pediatr* 2016; 83:1141-1149.
5. Reid ES, et al. Advantages and pitfalls of an extended gene panel for investigating complex neurometabolic phenotypes. *Brain* 2016 Sep 6. pii: aww221. [Epub ahead of print]. doi: 10.1093/brain/aww221.
6. Verma IC, et al. Screening for inborn errors of metabolism. *J Neonatol* 2005; 19:107-116.
7. Wolf A., et al. Single base-pair substitutions at the translation initiation sites of human genes as a cause of inherited disease. *Hum. Mutat* 2011, 32: 1137e1143.