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ORIGINAL ARTICLE

Evaluating the Utility of Next Generation Sequencing Technology in the Diagnosis and Prevention of Genetic Disorders in India, the Early Experiences

Udhaya Kotecha[1] · Ratna Puri[1] · Sunita Bijarnia[1] · Sudha Kohli[1] · Renu Saxena[1] · Ishwar Verma[1]

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Abstract To describe the utilization of Next Generation Sequencing technologies for genetic counseling and prenatal diagnosis. Ten families requested prenatal testing in view of previously affected offspring with genetically heterogeneous/hitherto undiagnosed disorders. Next generation sequencing was offered as a first tier investigation (1) in the probands who had not been diagnosed by baseline investigations, and (2) in cases where preliminary examination/testing suggested a genetically heterogeneous disorder, while the precise diagnosis was not available. The subsequently identified molecular basis enabled prenatal testing. The disorders included primary microcephaly, epidermolysis bullosa, inborn error of metabolism, infantile hypotonia, neuro-regression and sensorineural hearing loss. Five out of ten couples approached us during an ongoing pregnancy with two in the second trimester of gestation. Demise of the proband resulted in incomplete investigations in three cases. In seven cases, the disorder suspected was genetically heterogeneous and hence next generation testing was carried out while in the remaining three it was performed to ascertain the underlying gene involved. Fetal analysis detected recurrences in two cases, and the couples concerned decided to discontinue the pregnancies. Next generation sequencing proved a useful tool in select situations to overcome some of the challenges hindering a precise diagnosis in genetically heterogenous disorders.

✉ Ishwar Verma
jfm200@yahoo.in

[1] Institute of Medical Genetics and Genomics, Sir Ganga Ram Hospital, Rajinder Nagar, New Delhi 110060, India

Keywords Multi gene panel · Prenatal diagnosis · Autosomal recessive · Next gen sequencing

Introduction

The burden of genetic disorders is high in India, mainly due to the practice of consanguineous and endogamous marriages, in addition to the large number of births per year [1, 2]. However, genetic expertise is available only in a few centres and hence genetic work up of the cases is often incomplete [3]. In many families, the proband dies without a definitive diagnosis. Molecular analysis is often not pursued due to their cost as in India medical expenses are mainly borne by the family [4].

Requests for prenatal testing are very common in India, due to socio-economic and cultural factors [5]. A prerequisite for prenatal testing is identification of the underlying genetic etiology. Hence in many instances, fetal testing is not possible, leading to parental anxiety in subsequent pregnancies. Delayed referrals further compound the issue, as termination in India is permitted only till 20 weeks of gestation [6].

Many disorders are genetically heterogeneous. Sanger sequencing of various genes sequentially is expensive. Screening multiple genes together is now possible with Next Generation Sequencing (NGS), making it an attractive option for the same [7]. Feasibility has also improved as many Indian as well as Indian based foreign laboratories are offering NGS testing within the country. However, extensive pre and post test counseling is needed before offering this test, thus increasing the burden on genetic counseling services [8]. The current manuscript describes how utilization of NGS has eased prenatal diagnosis

in situations which hitherto were considered difficult due to the need of sequential testing via Sanger sequencing.

Cases and Methods

We describe ten families who had consulted us during 2014–2015 for prenatal testing at the Institute of Medical Genetics and Genomics, Sir Ganga Ram Hospital a tertiary care referral center at New Delhi, India. All the concerned families had a previous affected offspring with a suspected genetic disorder. However, in all cases, genetic analysis had either not been performed or mutations had not been identified in spite of extensive testing. NGS was offered as the disorder suspected on clinical examination was genetically heterogeneous in seven cases, whereas in the remaining three the disorder was undiagnosed.

In the pretest counseling session, the risk of recurrence, the need to identify the underlying causative mutation, the availability of NGS testing and its limitations were discussed. The need for validation, segregation analysis, and the possibility of identifying variants of unknown significance was stressed. After obtaining informed consent, the testing was outsourced to different collaborating laboratories. The respective laboratories performed NGS testing as well as data interpretation and variant calling. The results were then correlated with the clinical phenotype. The subsequent Sanger validation, segregation analysis and prenatal testing were performed in our laboratory.

Results

There were three cases of epidermolysis bullosa, two each of primary microcephaly and probable inborn errors of metabolism and one each of neuroregression, infantile hypotonia and congenital sensorineural hearing loss. Consanguinity was present in two cases, one with primary microcephaly and one with epidermolysis bullosa. Only five out of the ten families visited us in the pre-conceptional period, while the remaining five couples were referred after pregnancy was confirmed. In three of the five expectant mothers, there had been a demise of the proband, and the genetic work up was incomplete. Parental testing was resorted to in one case due to lack of availability of the proband's sample/tissue. These cases are briefly described below while results of molecular analysis as well as fetal testing are provided in Table 1.

Family 1 had a healthy daughter, followed by a son whose milestones had regressed after 1.5 years of age. The neuro-regression was preceded by an episode of fever and seizures which were myoclonic and refractory to therapy. He had been extensively investigated at the age of 2 years. An MRI revealed incomplete myelination and minor periventricular changes while the ophthalmologic examination was normal. A serum ammonia, lactate, uric acid, tandem mass spectrometry, chromosomes, methylation testing for Angelman syndrome and enzyme assays for infantile and late infantile neuronal ceroid lipofuscinosis, Krabbe disease, metachromatic leukodystrophy and GM2 gangliosidosis were all normal. The family approached us during their third pregnancy. A repeat MRI showed extensive cerebral atrophy and periventricular white matter changes. Considering infantile onset neuro-regression and the ongoing conception, the couple were advised the Illumina TruSight panel testing which analyzes 552 childhood onset recessive disorders.

Family 2 A neonatal death prompted genetic consultation. An uneventful pregnancy led to the birth of a floppy infant who needed ventilation. A sepsis screen, serum electrolytes, karyotype, tandem mass spectrometry, creatine kinase, SMA deletion, Prader Willi methylation analysis and congenital myotonic dystrophy testing were normal. A muscle biopsy revealed predominance of type 1 fibres along with central nucleation seen in some cells and variation in fibre size. The family history had been non-contributory and considering early onset disease we chose to perform the Illumina TruSight panel for inherited pediatric disorders.

Family 3 A male neonate had succumbed previously, and the family consulted us when they observed similar features in their second born. The clinical profile, as gleaned from the medical records (proband was not brought) showed encephalopathy, acidosis with pH 7.04, HCO 3:7.3 and BE-21.3, hyponatremia (S.Na:124 mEq/l) and hyperkalemia (S.K+:7.36 mEq/l). The 17 hydroxy progesterone, dehydroepiandrostenedione and tandem mass spectrometry were normal. Due to recurrence they were counseled about the possibility of a genetic disorder and advised to store a DNA sample in event of death to enable further studies. The couple returned later for preconceptional counseling. Illumina TruSight panel testing was performed on stored DNA.

Family 4 had lost two children in the neonatal period with a suspicion of propionic or methyl malonic academia. The diagnosis was based on the tandem mass spectroscopy report of the second child. In view of genetic heterogeneity as well as non-availability of DNA of the affected child, an organic acidemia panel testing was performed on one of the partners. This revealed a novel heterozygous variant of unknown significance in the MMAB gene. The couple were informed that prenatal testing was not recommended on the basis of these results as the variant may or may not be causative of disease. They however insisted on fetal testing and the same was performed along with metabolite analysis of amniotic fluid [9]. The results showed elevated

Table 1 Clinical phenotype, results of multigene panel testing and fetal testing in ten families

Sr. no and consanguinity	Clinical phenotype	Test performed	Gene	Molecular analysis/genotype	Diagnosis	Prenatal testing
Family 1: non consanguineous	Neuroregression	Illumina TruSight For pediatric onset recessive disorders	*GLB1* ENST00000307363 NM_000404.3	Compound Heterozygous c.1310delA (p.Asn437Metfs*24) c.1769G > A (p.Arg590His)	GM1 gangliosidosis	Fetus unaffected (normal)
Family 2: non consanguineous	Infantile hypotonia	Illumina TruSight For pediatric onset recessive disorders	*MTM1* ENST00000370396 NM_000252.2	*Hemizygous* c.1420C > T (p.Arg474Ter)	X-linked myotubular myopathy	Fetus unaffected (normal)
Family 3: non consanguineous	Inborn error of metabolism	Illumina TruSight For pediatric onset recessive disorders	*NDUFS6* ENST00000274137 NM_004553.4	Homozygous c.286C > T (p.His96Tyr)	Mitochondrial complex 1 deficiency	Fetus unaffected (carrier)
Family 4: non consanguineous	Inborn error of metabolism	Organic academia panel	*MMAB* ENST00000545712 NM_052845.3	Homozygous c.2T > G (p.Met1Arg)	Methylmalonic acidemia	Fetus affected
Family 5: consanguineous	Primary microcephaly	Primary microcephaly panel	*WDR62* ENST00000401500 NM_001083961.1	Homozygous c.1107del C (p.Ile370SerfsTer60)	Primary microcephaly	Fetus unaffected (normal)
Family 6: non consanguineous	Primary microcephaly	Primary microcephaly panel	*CENPJ* ENST00000381884 NM_018451.4	Compound Heterozygous c.2328dupA (p.Val777Serfs*3); c.3217-11_3217-10delTT	Primary microcephaly	Fetus unaffected (carrier)
Family 7: non consanguineous	Epidermolysis bullosa	Illumina TruSight For pediatric onset recessive disorders	*COL7A1* ENST00000328333 NM_000094.3	Compound Heterozygous c.7828C > T (p.Arg2610Ter); c.8541delT (p.Glu2848ArgfsTer50)	EB dystrophica	Fetus affected
Family 8: non consanguineous	Epidermolysis bullosa	Epidermolysis Bullosa panel	*COL7A1* ENST00000328333 *NM_000094*	Homozygous c.3958G > C (p.Gly 1320Arg)	EB dystrophica	Fetus unaffected (carrier)
Family 9: consanguineous (from Sudan)	Epidermolysis bullosa	Illumina TruSight For pediatric onset recessive disorders	*COL7A1* ENST00000328333 *NM_000094*	Homozygous c.8012G > A (p.Gly2671Glu)	EB dystrophica	Fetus unaffected (carrier)
Family 10: non consanguineous	Deafness	Deafness panel	*USH2A* ENST00000307340 NM_206933.2	Compound Heterozygous c.10510C > G (p.Pro3504Ala) c.7168G > A (p.Gly2390Arg)	Usher syndrome	Fetus unaffected (carrier)

levels of methylcitrate (10.26 nmol/ml, control-0.99 nmol/ml) and methylmalonate (45.86 nmol/ml, control-0.83 nmol/l) in the amniotic fluid and the molecular analysis revealed a homozygously affected fetus.

Families 5 and 6 consisted of two unrelated Indian families, whose affected children underwent primary microcephaly panel testing. The third-degree consanguineous couple in family 5 approached us at 12 weeks gestation during their third conception. Their first-born male had severe intellectual disability with a head circumference of 29 cm at birth. MR imaging had revealed non lissencephalic cortical dysplasia. In family 6, the proband had been delivered at term and the occipitofrontal circumference had been noted to be below 3rd centile suggesting primary microcephaly. At the time of examination, he was 5 years old, had learning difficulties but was attending a normal school.

Families 7, 8 and 9 Epidermolysis bullosa (EB) dystrophica was the clinical diagnosis in three patients, two non-consanguineous families from India and one consanguineous couple from Sudan. The probands' in families 7 and 8 had succumbed to disease. Presence of extensive blistering, desquamation and oral ulceration after birth led us to diagnose epidermolysis bullosa in family 7. A skin biopsy followed by immunostaining was advised as a guide to molecular analysis [10]. However, the family did not consent for the same. Phenotypic overlap between dystrophic and junctional variants was discussed and *COL7A1* analysis was recommended as a first step with panel testing as a second step. Direct Sanger sequencing of the *COL7A1* gene is expensive as the gene is very large (118 exons). The family opted instead for NGS panel testing for EB that included recessive genes for EB dystrophica and junctional type. In the second Indian couple (family 8) a skin biopsy had been performed on the proband suspected to have EB dystrophica-generalized severe type. Immunofluorescence based antigen mapping showed linear band with Collagen 7, mapping to the roof of the blister with a weak Laminin 5 band. The DNA of the affected child was extracted from the skin biopsy blocks and testing was conducted on the same. The third family (family 9) from Sudan was referred for prenatal analysis. Their previously affected son had EB dystrophica-generalized non severe variety. The gestational age at referral was 8 weeks. They also opted for a panel testing.

Family 10 This couple had previously consulted us for non-syndromic sensorineural hearing loss in their daughter. Testing of the *GJB2* gene, responsible for 17% of autosomal recessive deafness in India, revealed the common Indian mutation, p.Trp77* in heterozygous state [11, 12]. Further testing for mutations at the same locus, deletion and sequencing of *GJB6* was normal. A deafness panel study revealed a compound heterozygous variant in the *USH2A* gene. Fundus examination revealed retinal pigmentary changes, confirming the diagnosis of Usher syndrome [13, 14]. A previous eye evaluation 2 years of age had been normal, hinting development of retinal changes over time.

Discussion

The hurdles precluding prenatal testing in resource limited settings include incomplete investigations (family 3), unavailability of sample/tissue for further analysis following demise of the proband (family 4) and referral during an ongoing pregnancy (families 1, 4, 5, 9, 10) leaving little time for establishing the diagnosis and identifying the responsible pathogenic variations.

The above observations suggest that NGS testing can be considered an effective tool in both clinically undefined cases as well as in scenarios where clinical phenotyping is possible. If we consider primary microcephaly as an example (which was observed in family 5 and 6), there are currently 12 genes known to causative [15], Sanger analysis of ASPM, the most commonly involved gene reveals mutations in only 25–50% of case. Hence NGS, with the ability to sequence multiple genes together appears as an attractive option here.

NGS also offers the option to shift invasive testing as supportive second line testing. Traditionally, immunostaining after blister induction is the first step towards Epidermolysis bullosa molecular subtyping [16, 17]. These facilities are available only at select centres in India and conflicting results could be observed even after immunostaining like in case 8 where *COL7A1* staining was preserved. Genotype–phenotype correlation revealed the mutation to be in the triple helical domain where preserved staining is known, thus emphasizing the need of molecular analysis [18].

Diagnosis of mitochondrial disorders (like the one detected in family 3) has always been a challenge due to the large number of genes involved, non-specific nature of symptoms and the need for performing oxidative phosphorylation or respiratory enzymology. The latter two require invasive sampling and often have poor sensitivity. In India, where baseline testing like blood lactate, pyruvate, FGF-21, MRS as well as enzymology are not uniformly available, NGS can be viewed as a tool with tremendous potential in diagnosing mitochondrial disorders [19].

NGS testing is not without concerns. Detection of novel variations and finding multiple variations are both common in NGS testing. While on the basis of phenotype, it is easier to attribute pathogenicity to deletions, frameshifts and termination codons, start loss changes similar to the one

observed in family 4 pose a problem [20, 21]. Diseases causing variations due to loss of the initiation codon have been reported in cases of Cerulean cataract, Walker–Warburg syndrome but never in MMA [22, 23]. In the absence of functional studies, the results were deemed as "variants of unknown significance". Lack of the DNA of the affected child made it impossible to conclusively confirm the disease. However, it was possible to discuss prenatal testing as the baseline investigations in the proband had narrowed down the possibilities to two disorders. Since initiation codon mutations have never been reported before in MMA, we attempted to validate fetal testing both by molecular as well as metabolite analysis [24]. However, this approach of parental testing in the absence of the proband is not ideal and not typically recommended.

Variations in multiple genes causing the same phenotype is another common concern in NGS testing. This was observed in case 10 where the proband harbored mutations in both *GJB2* as well as *USH2A*. *GJB2* mutations are implicated in both autosomal recessive as well as dominant SNHL. However, the particular mutation c.231G > A (Trp77*) has been reported only in homozygous or compound heterozygous cases [14, 25]. Further, the presence of retinal pigmentary changes along with deafness is consistent with the diagnosis of Usher syndrome. The need to repeat ancillary tests to achieve a clinical correlation is highlighted in this case as some findings appear late [16].

To conclude, this small series exemplifies how next generation sequencing is a promising tool for diagnosis of genetically heterogenous disorders. However, it is important to note that diagnostic utility is influenced by clinical phenotyping, as this affects choice of panel and is necessary for appropriate genotype–phenotype-co-relation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interests.

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