Original Article

Next generation sequencing in Western Indian children with nephrolithiasis and/or nephrocalcinosis: An observational study

Anshuman Saha[1], Kinnari B. Vala[1], Himanshu V. Patel[2]

From [1]Assistant Professor, Department of Pediatric Nephrology, Institute of Kidney Diseases and Research Centre and Institute of Transplantation Sciences, Ahmedabad, Gujarat, India, [2]Professor, Department of Nephrology, Institute of Kidney Diseases and Research Centre and Institute of Transplantation Sciences, Ahmedabad, Gujarat, India

ABSTRACT

Background: About half of children with nephrolithiasis and/or nephrocalcinosis (NL/NC) have an underlying metabolic cause. Next generation sequencing (NGS) is increasingly being used as a clinical tool in diagnosing inherited renal diseases. **Objective:** The objective of the study was to retrospectively analyze the utility of NGS in diagnosis and management of children with NL/NC at a tertiary care referral nephrology center in western India. **Methods:** All children ≤18 years with NL/NC, where a NGS was sent from September 2016 to September 2020, were included in the study. Clinical exome sequencing covering 8882 genes was done in 13 children. The test result was interpreted as per the American College of Medical Genetics classification: No pathogenic variant, variant of unknown significance (VUS), and likely pathogenic and pathogenic variant. **Results:** The median age (IQR) of the cohort at onset of disease was 11 months (4.25–102). History of consanguinity was present in 3 children (23%). Eight children had NC 7 had NL, 7 had end stage renal disease (ESRD) at presentation. Seven out of 8 children with NC had ESRD. Eleven children (84.6%) had an identifiable monogenic genetic cause: 9 had pathogenic, 2 had likely pathogenic variants detected in 5 genes: AGXT, GRHPR, HOGA1, CLDN16, and HPRT1. Two VUS were detected in two children in BBS4 and KCNJ1 gene. Primary hyperoxaluria (PH) Type 1 was the most common diagnosis in 6 children. Other diagnoses were Lesch–Nyhan syndrome in 2, PH 2, PH3, and FHHNC in 1 each. **Conclusion:** The yield of NGS in children with NL/NC was remarkably high. NGS was useful in diagnosis and management of children with NL/NC.

Key words: Indian children, Nephrocalcinosis, Nephrolithiasis, Next generation sequencing

Nephrolithiasis (NL) is a major public health issue in adults particularly in arid climates [1]. NL has traditionally been considered a disease of adults; however, the prevalence in children is increasing worldwide [2,3]. About half of children with a kidney stone have a metabolic cause [4]. Even a single stone in children in absence of obvious risk factors should raise the suspicion of underlying metabolic cause and prompt thorough evaluation [5]. Nephrocalcinosis (NC) in presence of NL with or without renal dysfunction is an important clue of an underlying metabolic etiology [6]. Detail biochemical evaluation including a 24 h urine sample lead to identification of underlying etiology in most of the metabolic causes. However, majority of these diseases are inherited and have an underlying monogenic mutation which not only establishes diagnosis unequivocally but also guide in specific management [6].

A recent study identified pathogenic disease causing mutation in a single gene in 21% of 106 children with a previously undetermined etiology, indicating its potential clinical utility in the care of these patients [7]. To date, more than 30 genes have been implicated in NL or NC, with autosomal-dominant, recessive, and X-linked inheritance [8]. We aimed to analyze the utility of next generation sequencing (NGS) in diagnosing suspected genetic cause of NL and/or NC in western Indian children.

Access this article online	
Received - 15 February 2021 Initial Review - 03 March 2021 Accepted - 11 March 2021	Quick Response code
DOI: 10.32677/IJCH.2021.v08.i04.002	

METHODS

Children under the age of 18 years with NL with or without NC, where a next NGS was sent from September 2016 to September 2020 were included for retrospective analysis after approval of institutional ethics committee. Five milliliters of blood in ethylenediaminetetraacetic acid were sent from the patient after obtaining written informed consent to Medgenome Labs Pvt. Ltd., a private commercial laboratory at Bengaluru, India. Demographic details, history of consanguinity, similar illness in family, early onset of end stage renal disease (ESRD), involvement

Correspondence to: Anshuman Saha, Department of Pediatric Nephrology, Institute of Kidney Diseases and Research Centre and Institute of Transplantation Sciences, Ahmedabad, Gujarat, India. E-mail: drasaha1980@gmail.com

of multiple systems, and syndromic features were noted. Children with a clinical picture of tubulopathy and NC were excluded from the study.

Clinical exome sequencing (CES) covering 8332 disease causing genes encompassing both nuclear and mitochondrial genes was done in index patient. It covered disease associated genes including coding variants, splice variants, reported deep intronic variants, and copy number variation. Sanger sequencing was done when sibling or any other family member was to be tested for the same gene or to confirm the variant in NGS. The test result was interpreted as per the American College of Medical Genetics classification: no pathogenic variant, variant of unknown significance (VUS), and likely pathogenic and pathogenic variant [9].

Targeted Gene Sequencing

Selective capture and sequencing of the protein coding regions of the genome/genes were performed. Mutations identified in the exonic regions are generally actionable compared to variations that occur in non-coding regions. Targeted sequencing represents a cost-effective approach to detect variants present in multiple/large genes in an individual. DNA extracted from blood was used to perform targeted gene capture using a custom capture kit. The libraries were sequenced to mean >80–100X coverage on Illumina sequencing platform.

The sequences obtained were aligned to human reference genome (GRCh37/hg19) using Burrow Wheeler Aligner program [10,11] and analyzed using Picard and Gene analysis tool kit (GATK) version 3.6 [12,13] to identify variants relevant to the clinical indication. The GATK best practices framework was followed for identification of variants in the sample. Gene annotation of the variants was performed using variant effect predictor program [14] against the Ensembl Release 87 human gene model [15].

Clinically relevant mutations were annotated using published variants in literature and a set of diseases databases – ClinVar, Online Mendelian Inheritance In Man, Genome Wide Association Study, Human Gene Mutation Database, and SwissVar [16-22]. Common variants are filtered based on allele frequency in1000 Genome. Phase 3, Exome Aggregation Consortium, Exome Variant Server, Single Nucleotide Polymorphism (dbSNP147), 1000 Japanese Genome and our internal Indian population database [23-27].

Nonsynonymous variants effect was calculated using multiple algorithms such as Polymorphism Phenotype version-2 Sorting Intolerant from Tolerant, Mutation Taster-2, Mutation Assessor, and Likelihood Ratio Test. Only non-synonymous and splice site variants found in the clinical exome panel consisting of 8332 genes were used for clinical interpretation. Silent variations that do not result in any change in amino acid in coding region were not reported.

RESULTS

During the study period, 83 samples were sent for NGS from 72 children, 8 parents, 1 sibling, and 2 fetuses. Of 72 children, 13 (18%) had NL with or without NC. CES was sent in all 13 children (7 female and 6 male) and 2 fetuses. The median age (IQR) of the cohort at onset of disease was 11 months (4.25–102). Median age (IQR) at diagnosis was 12 months (6–180). History of parental consanguinity was present in 3 children (23%). Family history of similar illness in a sibling was present in 1 child.

Eight children (61.5%) had NC. Seven (87.5%) children with NC had pathogenic mutation. All those seven children had ESRD. Seven children presented in infancy. Three of them presented with anuria, hyperechoic kidneys, and when renal biopsy was done to find out etiology, had oxalate crystals in biopsy. Two infants had obstructive acute kidney injury (AKI) due to NL and hyperuricemia. Three children had ESRD and dense NC. One child presented with stage 4 chronic kidney diseases (CKD), NC, and hypomagnesemia. One child had recurrent urinary tract infection (UTI) and on ultrasonography was found to have dense NC. Only 1 child had normal renal function at presentation.

Nine children had a pathogenic variant, 2 likely pathogenic, and 2 had VUS. Therefore, 11 children (84.6%) were diagnosed to have a genetic cause for NL/NC. Inheritance was autosomal recessive in 9 (81%) and X linked recessive in 2 children. Fifteen pathogenic variants in 11 children were detected in five genes: Alanine–glyoxylate transferase (AGXT), hypoxanthine phosphoribosyl transferase 1 (HPRT1), 4 hydroxy 2 oxoglutarate aldolase 1 (HOGA1), glyoxylate and hydroxy pyruvate reductase (GRHPR), and claudin (CLDN16P). Four (30.7%) were novel variants not described earlier. Nine were missense, 2 frameshift, 1 splice site, and 1 was truncating mutation. Two VUS were detected in two children in Bardet–Biedl syndrome 4 (BBS4) and Potassium Inwardly Rectifying Channel Subfamily J Member 1 (KCNJ1 gene).

Primary hyperoxaluria (PH) was the most common diagnosis in 8 children followed by Lesch–Nyhan syndrome in 2, familial hypomagnesemia hypercalciuria with nephrocalcinosis (FHHNC) in 1. Clinical and genetic details of patients with genetic disease are given in Table 1.

Out of eight children with PH, six had pathogenic variant in AGXT gene (PH Type 1), 1 in GRHPR (PH Type 2), and 1 in HOGA1 (PH Type 3). Five children with PH Type 1 presented in infancy, youngest at 3 months. Three of them presented with anuria, required peritoneal dialysis. All of them had echogenic kidneys in ultrasonography and on kidney biopsy had oxalate crystals in tubules. All of them progressed to ESRD and expired within 3 months of follow-up.

One child presented with ESRD at 10 years of age without any prior history of NL and got diagnosed to have PH Type 1 by NGS after 2 years of presentation while undergoing evaluation for renal transplantation. He died waiting for liver kidney transplantation. One patient presented at 25 years with ESRD. Kidney biopsy was done to find out etiology which revealed oxalate crystals. On further history taking, he was having NL for 4 years of age. The child with pathogenic variant in HOGA1, presented with multiple renal calculi with normal renal function. The child with GRHPR presented with ESRD at 12 years of age with history of NL in

Table 1: Details of patients with nephrolithiasis and nephrocalcinosis

Age in months	Clinical presentation	Clinical diagnosis	Gene, location	Codon change, protein change	Zygosity	Inheritance	Final Diagnosis	Outcome
5	Anuria, AKI, NC, oxalate in biopsy, progressed to ESRD	PH	AGXT Exon 2	c. 245G>A p.Gly82Glu	Homozygous	AR	PH 1	Confirmed diagnosis
3	ESRD, NC	PH	AGXT, Exon 1	c. 32C>G, c. 107G>A p.pro11Arg p.Arg36His	Homozygous	AR	PH 1	Confirmed diagnosis
10	NL, obstructive AKI, hyperuricemia	Uric acid NL	HPRT1, Exon 3	c. 212dupG p.Tyr72LeufsTer2	Hemizygous	XLR	Lesch–Nyhan syndrome	New diagnosis
1	NL, obstructive AKI, Hyperuricemia	Uric acid NL	HPRT1, Intron 1	c. 27+2T>G 5'slice	Hemizygous	XLR	Lesch–Nyhan syndrome	New diagnosis
12	Nephrolithiasis	PH	HOGA 1 Exon 1	c. 134C>T p.Pro45Leu	Homozygous	AR	PH 3	Confirmed diagnosis
6	Anuria, AKI, oxalate crystal in biopsy, progressed to ESRD	PH	AGXT Exon 2	c. 302T>C p.Leu101Pro	Homozygous	AR	B	Confirmed diagnosis
25 year	CKD	PH	AGXT Exon 1,2	c.c. 33dupC c. 245G>A p.Lys12GlnfsTer156 p.Gly82Glu	Homozygous	AR	PH 1	Confirmed diagnosis
12 year	ESRD	PH	GRHPR Exon 4	c. 349T>C p.Ser117Pro	Homozygous	AR	PH 2	Confirmed diagnosis
12 year	Nephrocalcinosis, CKD	FHHNC	CLDN16 Exon 2	c. 374T>C p.Phe125Ser	Homozygous	AR	FHHNC	Confirmed Diagnosis
16 year	NL, CKD, coarse facies	Unknown	BBS 4 Exon 11	c. 760G>A p.Val254Ile	Homozygous	AR	Bardet–Biedl syndrome	VUS
12 year	ESRD	PH	AGXT Exon 1,5	c. 33dupC, c. 577dupC p.Lys12GlnfsTer156 p.Leu193ProfsTer32	Compound heterozygous	AR	PH 1	Confirmed diagnosis
4	ESRD	PH	AGXT Exon 2	c. 245G>A p.Gly82Glu	Homozygous	AR	PH 1	Confirmed diagnosis
10 year	NC	PH	KCNJ 1 Exon 2	c. 658C>T p.Leu220Phe	Homozygous	AR	Bartter syndrome 2	VUS

AKI: Acute kidney injury, ESRD: End stage renal disease, CKD: Chronic kidney disease, NL: Nephrolithiasis, NC: Nephrocalcinosis, PH: Primary hyperoxaluria, FHHNC: Familial hypomagnesemia hypercalciuria with nephrocalcinosis, AGXT: Alanine–glyoxylate transferase, CLDN 16: Claudin 16, BBS4: Bardet–Biedl syndrome 4, HPR1: Hypoxanthine guanine phosphoribosyl transferase 1, GRPHR: Glyoxylate reductase/Hydroxypyruvate reductase, KCNJ1: Potassium inwardly rectifying channel subfamily J member 1

past. Erect X-ray abdomen revealed calcified kidneys suggesting oxalosis.

Two children, one at 1 month and other at 3 months of age presented with obstructive AKI due to NL and accompanying UTI. Both had hyperuricemia and diagnosed to have Lesch–Nyhan syndrome. One of them had cleft lip and palate along with NL. Both have normal renal function after nephrolithotomy and have normal uric acid level on allopurinol. One 15-year-old girl presented with CKD stage 4 and history of NL from early childhood. On evaluation, there was hypomagnesemia, hypercalciuria, and NC. Her younger brother also had similar symptoms. She had pathogenic variant in claudin 16 confirming the diagnosis of FHHNC.

There were 2 VUS in BBS4 and KCNJ1 in two children. The girl with VUS in BBS4 presented with NL, CKD stage 4, and coarse facial features. The other girl presented with recurrent UTI. Routine renal ultrasound showed dense medullary NC. Twenty-four-hour urine examination revealed hypercalciuria. She was suspected to have PH. However, to our surprise she had VUS in KCNJ1 which causes Bartter syndrome Type 3. There was no history of polyuria or polydipsia. Evaluation revealed metabolic alkalosis (HCO_3 27 mEq/l), low normal serum potassium (3.2mEq/l), normal serum sodium (135 mEq/l), and chloride (103 mEq/l). Urine chloride was high (62 mEq/l). Phenotypically, she fitted into barter phenotype. Renin and aldosterone level has been planned.

After confirming genetic diagnosis in two children, one with AGXT and one with HPRT1 Sanger sequencing in fetal chorionic villus sample was done in next pregnancy and the fetuses were aborted after the same mutations were detected in the fetuses.

DISCUSSION

We discuss the results of CES done by NGS in children with NL/NC at a tertiary care referral nephrology center in western India. There is no published data yet on NGS in Indian children with NL/NC. About 30 genes have been discovered to cause NL and NC [8]. All those, disease-causing genes were tested by CES

in our cohort. It also included tubulopathy and ciliopathy genes that may have phenocopy of NL, but were excluded from this analysis as children suspected with distal tubular renal acidosis (dRTA), Bartter syndrome, other tubulopathies, and suspected ciliopathies were excluded from this analysis.

Extremely high proportion (84.6%) of children had a pathogenic variant, which is way high compared to 20% and 30% in earlier cohorts [7,28]. This was also higher than reported in other diseases such as steroid-resistant nephrotic syndrome (26%), congenital anomalies of kidney and urinary tract (17%), NL/NC (25%), and ciliopathies (50%) [29]. Fourteen genes accounted for 15% of NL/NC in a mixed population of children and adults, where the proportion of monogenic disease in children was 20% [7]. The same group subsequently reported 9 genes accounting for monogenic disease in 30% of subjects under 25 years of age by whole exome sequencing covering 117 genes [28]. The high proportion in our cohort could be because NGS was done on subjects whose pre-test probability was already very high unlike other studies where all children with NL were tested.

NC is associated with ESRD [6] and should be a clue toward presence of underlying genetic disease. In our cohort also, one child with NC had ESRD at presentation. Children with NL and chronic renal dysfunction are usually diagnosed with any of the five diseases: PH, adenosine phosphor ribosyl transferase deficiency, cystinuria, dent disease, and FHHNC and should be evaluated for the same [30]. Ramya *et al*. reported etiology of NC in 54 children managed at a single tertiary care center in South India. The proportion of dRTA and PH was 33.3% and 16.7%, respectively. Many children had hypercalciuria secondary to RTA, Dent syndrome, Bartter syndrome, and hypomagnesemia. However, genetic confirmation was sought in only 8 children [31].

Eight of 11 children with genetic disease in our cohort had PH. Six were diagnosed with PH Type 1, 1 PH Type 2, and 1 PH Type 3. Children with PH Type 1 had more severe presentation. All of them had ESRD at presentation. Four of them presented in infancy and three succumbed to illness. PH1 has more severe presentation and poorest prognosis among the three types of PH [32]. Three of the infants with PH1 presented with anuria at presentation. Anuria in the presence of normal size but very hyperechoic kidneys suggesting NC in infants should raise the suspicion of PH1. However, the presentation can be variable as in two of our children and they can be presented in late childhood or adulthood without any history of NL in past [32]. One got diagnosed at 25 years of age and other at 12 year of age. The important lesson here is to keep a high index of suspicion when the etiology of CKD is unclear or in presence of hyperechoic kidneys suggesting NC.

Genetic testing is the only test that can provide precise molecular diagnosis in patients with PH. The clinical presentation and natural history of disease are determined by the genetic mutations. PH Type 2 and 3 are less severe and require only kidney transplantation whereas PH Type 1 will require combined liver and kidney transplantation [32]. Further, patients with specific AGXT mutationse.gp.Gly170Arg orp.Phe152Ile, respond well to oral pyridoxine [33]. Hence, a precise diagnosis by NGS must be sought in suspected PH patients. Precise genetic mutation also helps in prognosticating the disease and genetic counseling, as happened in detection of same mutation in AGXT gene in a fetus in subsequent pregnancy and the fetus was aborted.

A common missense mutation in exon 2 which causes substitution of thymidine with cytosine at nucleotide 302 (c.302T > C) has earlier been reported in three children of north western subcontinent of India and Pakistan suggesting a common mutation in Indian population [34]. We found the same variant in one child. However, the same variant was not reported in a South Indian cohort of 7 children [35]. The variants in our cohort were different than previous two cohorts [34,35]. We observed pathogenic variant in exon 2 which caused substitution of guanosine by adenine at nucleotide 245 (c.245G > A) in three children. Two of them presented in infancy with ESRD and succumbed to illness whereas the other got diagnosed at 25 years. The patient who was diagnosed at 25 years also had a duplication of cytosine at nucleotide 33 (c.33dupC) in exon 1 of AGXT gene. The variant in exon 2 (c.245G > A) could be a common disease-causing variant in West Indian population.

The child who got diagnosed with PH Type 2 presented with ESRD at 12 years of age with NL in past. The variant was a novel one and has not been reported earlier. The presentation in PH Type 2 is less dramatic compared to PH Type 1 but can cause serious morbidity and mortality. There are reports that liver transplantation can be avoided in PH Type 2 and only kidney transplantation can suffice, however, this require further verification [32]. One child had a pathogenic variant in HOGA 1 gene confirming diagnosis of PH Type 3. She had obstructive NL without NC and normal renal function. Stone analysis revealed calcium oxalate crystals. However, precise diagnosis of PH Type 3 could only be established because of CES. PH Type 3 has less severe presentation as in our case [32].

Two infants, one at 1 month and one at 3 month of age presented with obstructive renal calculi and hyperuricemia and got diagnosed with Lesch–Nyhan syndrome due to pathogenic variant in HPRT1 gene. One child had a novel pathogenic 5` splice site variant (c.27 + 2T > G) in intron of HPRT1 gene. The other child had pathogenic mutation in exon. Both patients are doing well on allopurinol without recurrence of obstructive stone and normal renal function. Both presented very early in life and did not have neurologic manifestation at presentation.

HPRT-related gout and Lesch–Nyhan syndrome are caused by mutations in the HPRT1 gene. The complete deficiency of HPRT leads to Lesch–Nyhan syndrome, whereas partial deficiency (at least 8%) is associated with the Kelley–Seegmiller syndrome. Lesch–Nyhan syndrome is characterized by abnormal metabolic and neurologic manifestations. In contrast, renal stones, uric acid nephropathy, and renal obstruction are often the presenting

symptoms of Kelley–Seegmiller syndrome, but rarely of Lesch–Nyhan syndrome [36].

One interesting child presented with recurrent UTI and dense medullary NC who was suspected to have PH was found to have a VUS in KCNJ1 gene to our surprise. The variant causes antenatal barter syndrome [37] but has been reported to cause a very mild Bartter phenotype in adults [38]. The presence of hypomagnesemia and hypercalciuria in a child with NC and CKD lead us to suspect FHHNC, which was confirmed by detection of pathogenic variant in CLN16. This also led to the same diagnosis in her sibling who had similar presentation. FHHNC is an autosomal recessive disease due to pathogenic mutation in CLN16 and claudin 19 genes [39]. The claudins are paracellular proteins involved in the paracellular transport of magnesium in thick ascending limb of loop of Henle. Involvement of claudin 19 also leads to ocular involvement causing myopia [38].

The major strength of our study is the novelty of NGS data in Indian children with NL and or NC. However, retrospective design of the study involving few children is the major limitation. Large multicentre prospective studies in future will be more informative.

CONCLUSION

NGS provided precise diagnosis in our cohort which would not have been possible otherwise. It helped in explaining prognosis and genetic counseling. The yield of NGS was very high, suggesting that when done in a well phenotyped patient the result is more useful.

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Funding: None; Conflicts of Interest: None Stated.

How to cite this article: Saha A, Vala KB, Patel HV. Next generation sequencing in Western Indian children with nephrolithiasis and/or nephrocalcinosis: An observational study. Indian J Child Health. 2021; 8(4):144-149.