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Genotype first approach & familial segregation analysis help in the elucidation of disease-causing variant for fucosidosis

FUCA1 gene codes for alpha-L-fucosidase enzyme and pathogenic variations (missense, nonsense, frameshift and splice site) are known to disrupt its catalytic function, leading to an autosomal recessive disorder (lysosomal storage disorder) called fucosidosis[1,2]. Absence of this enzyme or its reduced activity in fucosidosis leads to impaired degradation of both fucosylated glycoproteins as well as glycolipids in lysosomes, leading to the deposition of fucosylated substrates (>20) in different tissues. Variable symptoms of fucosidosis such as psychomotor deterioration, skin and skeletal abnormalities, growth retardation and intellectual disability have been reported[3]. Fucosidosis is classified into two types based on phenotype. Type 1 fucosidosis is characterized by a rapid psychomotor regression with a severe neurologic involvement at an early age leading to death usually within the first decade of life, whereas type 2 fucosidosis is characterized by milder neurologic signs, psychomotor retardation and a longer survival. In severe cases, symptoms typically show up in infancy, but the affected individuals usually live into their late childhood[4]. Till date, 160 variants are reported in *FUCA1* gene in ClinVar[5], of which 23 are pathogenic and 125 are variants of uncertain significance (VOUS). There are two reports from India regarding pathogenic variations in *FUCA1*[6,7]. Here, we report on genetic analysis in a case of syndromic psychomotor retardation and emphasize on importance of genotype first approach and utility of exome sequencing for detecting the copy number variants.

Two siblings, a girl of nine years and a boy of seven years of age, born out of a non-consanguineous union from Goa, India, presented to a neurologist with a history of developmental delay and language regression followed by non-verbal communication. There was no paternal or maternal family history of neurodegenerative disorders. Both patients had an uneventful perinatal history. Developmental delay was noticed from infancy in both children; however, some language in the form of single words had developed by 2.5-3 yr. However, language milestones regressed by seven years in the girl and five years in the boy. At present, both could only make sounds and point to indicated needs. There were dysmorphic facies with hypertelorism, depressed nasal bridge, low set ears, and tongue thrust (Fig. 1A). Anthropometric examination revealed stunting with failure to thrive, but head circumference was within normal range. Central nervous system examination revealed double hemiplegia, psychomotor retardation and cognitive decline in both patients; however, formal IQ testing was not done. Easy bruisability and petechiae-like rash were observed on the trunk of boy for the last 2-3 months (Fig. 1B). The girl had a fall from a high chair in 2018, which caused subluxation on C1-C2. She was treated conservatively after which she developed severe kyphosis of the spine. No seizures or cherry red spot was reported. Haematological investigations including platelet count and bleeding time were normal in both. Skeletal radiographs revealed no changes in the dysostosis multiplex. Magnetic resonance imaging of the brain exhibited hyperintense signal changes on T1-weighted imaging and showed hypointense signal changes on T2-weighted imaging in periventricular and bilateral globus pallidus regions, suggestive of hypoxic changes in both the siblings.

Whole blood samples (2 ml) in EDTA were collected from the affected siblings and their parents after taking a written informed consent. The genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, USA). The study protocol was approved by the Institutional Ethics Committee of Centre for DNA Fingerprinting & Diagnostics, Hyderabad (IEC 31/2019).

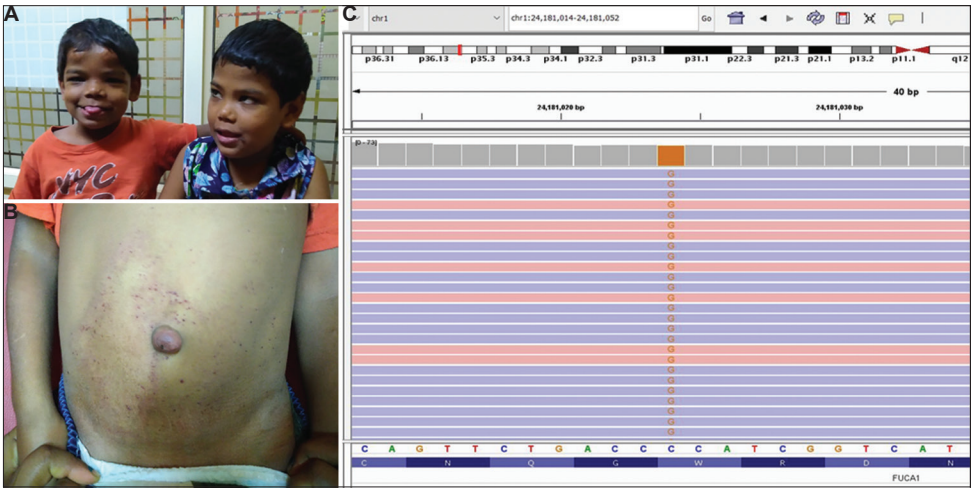

Fig. 1. Genotype–phenotype correlation and Sanger sequencing and RT-PCR validation of patient. (**A**) Facial photograph of both siblings showing dysmorphic facies. (**B**) Easy bruisability and petechiae-like rash observed on the trunk of proband. (**C**) IGV view of the BAM file showing the coordinate of *FUCA1*:c.795G>C:p.Trp265Cys variant identified using BWA-GATK pipeline from the whole exome NGS data. BWA, Burrows-Wheeler Aligner; GATK, genome analysis tool kit.

Whole exome sequencing of the DNA was performed using the Illumina platform (Illumina, San Diego, CA, USA) as per the manufacturer's protocol for the male proband. The sequences were analyzed using the bioinformatics pipeline described earlier[8], (details under Supplementary material online). After correlating with the clinical diagnosis, a novel homozygous NM_000147.5 (*FUCA1*_v001):c.795G>C, p.(Trp265Cys) (Depth: 49X) variant was identified in exon 5 of the *FUCA1* gene (Fig. 1C). This homozygous missense variant of the *FUCA1* gene had a combined annotation-dependent depletion (CADD) score of 31 and was predicted to be disease causing by the Mutation Taster and SIFT, *in silico* pathogenicity prediction programmes (Supplementary Fig. 1). This variant has not been reported previously in 1000 Genomes Project, gnomAD or ClinVar, human gene mutation database (HGMD®) databases. dbSNP database mentions c.795G>T variation at low frequency which also codes for the same amino acid change p.Trp265Cys. The variant was classified as 'Likely Pathogenic' based on the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines[9]. The presence of c.795G>C variant was checked in the female sibling and both parents using targeted PCR amplification and Sanger sequencing of exon 5 of the *FUCA1* gene. The sibling was homozygous while the father was heterozygous for the c.795G>C variant (Fig. 2A). Mother's DNA showed the presence of wild-type nucleotide at position c.795 (Fig. 2A). The variant was submitted in ClinVar (Submission Id: SUB11112585).

Quantitative PCR (qPCR) assay for *FUCA1* relative gene dosage for exon 5 compared to a reference gene (GAPDH) revealed heterozygous deletion in mother and both the probands (Fig. 2B). The heterozygous deletion was absent in father and two unrelated controls. The same result was verified using the read count distribution in 10 unrelated samples of whole exome sequencing data in exon 5 of *FUCA1* gene along with the male proband data. Results showed significant low read count in comparison with the 10 control samples in the exome data of proband (Supplementary Fig. 2). We could not ascertain the exact breakpoints of the deletion from exome sequencing data since the breakpoints are likely to be in introns.

In our case, the phenotype in the patient was not matching any particular condition. Hence, we planned for a genotype first approach and conducted exome sequencing in one of the affected. We identified an apparent homozygous novel missense likely disease-causing variant in the *FUCA1* gene that results in an amino acid change from tryptophan to cystine at the 265th position (p.Trp265Cys) of the alpha-L-fucosidase protein. The missense variant is likely to lead to change in protein structure or interfere with catalytic function of protein, which in turn will lead to deficiency or complete loss of function of fucosidase enzyme. Following this, we did reverse phenotyping and found that the clinical features in both the affected siblings were consistent with a diagnosis of fucosidosis. The phenotype was consistent with Type II fucosidosis in both siblings.

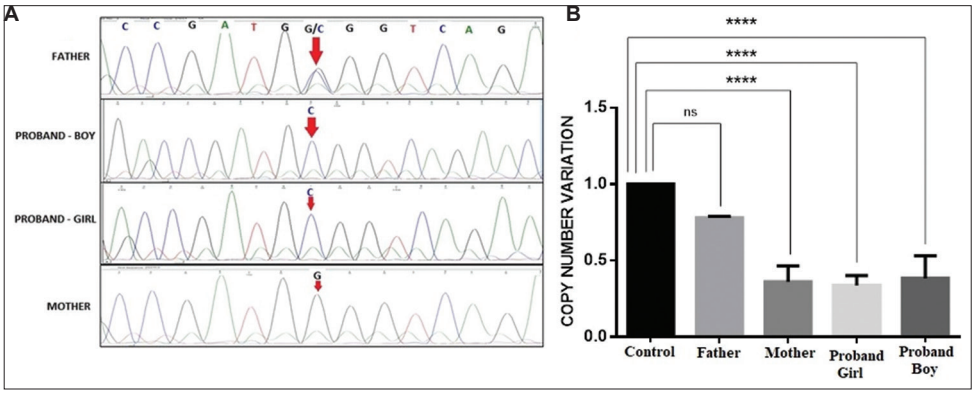

Fig. 2. (**A**) Electropherograms of the siblings and both parents. The c.795G>C (p.Trp265Cys) variant is indicated by the arrows. It was present in the siblings and father but absent in mother; (**B**) qPCR assay showing copy number variation of patients with respect to unrelated control. *GAPDH* was used as the reference gene. Tukey's multiple comparison test was employed to calculate the significance (P<0.05)ns: not significant, qPCR, quantitative PCR.

Familial segregation analysis in our case revealed the mother to be homozygous for wild-type allele. This was in contradiction to the expected heterozygosity in both parents for this autosomal recessive disorder. This phenomenon can be explained by one of the three reasons: (*i*) uniparental disomy resulting in homozygous variant in proband and only one parent being heterozygous for same variant; (*ii*) disputed paternity when father is found to be homozygous for wild type allele, or (*iii*) a heterozygous deletion of exon 5 in mother. Uniparental disomy and disputed paternity were unlikely in our case in view of recurrence in sibling. Hence, we hypothesized that the maternal allele carried a large deletion including exon 5, which can result in apparent homozygous wild-type allele genotype on Sanger sequencing. qPCR analysis confirmed our hypothesis and revealed normal dosage of exon 5 in father and heterozygous deletion in mother and both affected siblings. This reiterates the importance of familial segregation analysis following variant identification in exome/genome sequencing.

Our report expands the genotypic spectrum of fucosidosis and reiterates regarding need of genotype first approach in genetically heterogeneous conditions. Psychomotor retardation is a heterogenous clinical presentation, and often, no specific clinical diagnosis is apparent. Genotype first approach reduces the time to diagnosis in some cases. Family segregation studies are important even in apparently homozygous variants, as unusual phenomenon like deletion of one allele can be seen. Further genetic studies such as qPCR can help in ascertaining these rare instances. Reverse phenotyping, especially with involvement of a clinical geneticist, is important to correlate molecular findings with the phenotype.

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Supplementary Material

Methodology

Whole exome sequencing (WES) of the DNA was performed using Illumina platform. (Illumina, San Diego, CA, USA) following the manufacturer's protocol for the male proband. The exome library TruSeq RNA Library Prep Kit v2 (Illumina Inc., USA) was sequenced to >100× coverage on Novaseq platform (Illumina Inc., USA).

The sequences obtained were aligned to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner[1] programme and analysed using the Picard and Genome Analysis Tool Kit-Lite toolkit[2] to identify variants in the whole exome relevant to the clinical indication as described earlier[3].

Variant annotation was completed using ANNOVAR[4] for position and predicted function. Variant filtering was done using 1000 genomes[5], exome variant server, exome aggregation consortium[6] (using a minor allele frequency of ≤0.01) and dbSNP databases[7]. Clinically relevant variations were annotated using published variants in the literature and a set of variant databases including ClinVar[8], OMIM[9] and the Human Gene Mutation Database (HGMD)[10]. Following identification of the *FUCA1* variant through WES, targeted polymerase chain reaction (PCR) amplification was performed using exon -specific primers for exon 5 of the *FUCA1* gene, and Sanger sequencing was done using ABI 3130 Genetic analyzer (Life Technologies, Carlsbad, California, USA).

The information regarding WES raw data analysis is outlined in Supplementary Fig. 1. The VCF file had 138,157 number of variants and after stringent filtering of variants for combined annotation-dependent depletion (CADD)[11] score ≥20 left 218 homozygous variants, in which 10 are located in the exonic region of the genome. Following correlation with the clinical diagnosis, a novel homozygous NM_000147.5(*FUCA1*_v001):c.795G>C, p.(Trp265Cys) (Depth: 49X) likely pathogenic variant was identified in the *FUCA1* gene in exon 5 (Fig. 1C). This homozygous missense variant of the *FUCA1* gene has a CADD score of 31 and is predicted to be disease-causing by the Mutation Taster and SIFT, *in silico* pathogenicity prediction programmes. This variant has not been reported previously in 1000 Genomes Project, gnomAD, or ClinVar, HGMD databases (Supplementary Fig. 1). The variant can be classified as Likely Pathogenic (PM2, PM3, PP1, PP3) as per the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines[6]. The presence of c.795G>C variant was checked in the female sibling and both parents using targeted PCR amplification using F: 5′-ACCAAATGTCCCCTCCAGAG-3′ and R: 5 AACCAACACCGTTAAAGCCC-3′ primers and Sanger sequencing of exon 5 of the *FUCA1* gene. The sibling was homozygous while the father was heterozygous for the c.795G>C variant (Fig. 2A). Mother's DNA showed normal wild type variant (Fig. 2A).

A. Data & alignment statistics

Total no. of reads	21,21,94,197
Average input read length	35-100
Mapped reads	21,17,58,759
Alignment percentage	99.79%

B. Variant filtering strategy

Filtering criteria	No. of variants
Total no. of variants	138157
Rare variants (MAF <=0.01)	8704
No. of variants in homozygous state	406
Homozygous variants having CADD score >=20	218

C. Variant Interpretation

Gene (Transcript)	Location	Variant	Zygosity	Classification*	Disease (OMIM)	Inheritance
FUCA1 (NM_000147)	Exon 5	c.795G>C:p.Trp265Cys	Homozygous	Likely Pathogenic	FUCOSIDOSIS (#230000) α-L-Fucosidase Deficiency	Autosomal recessive

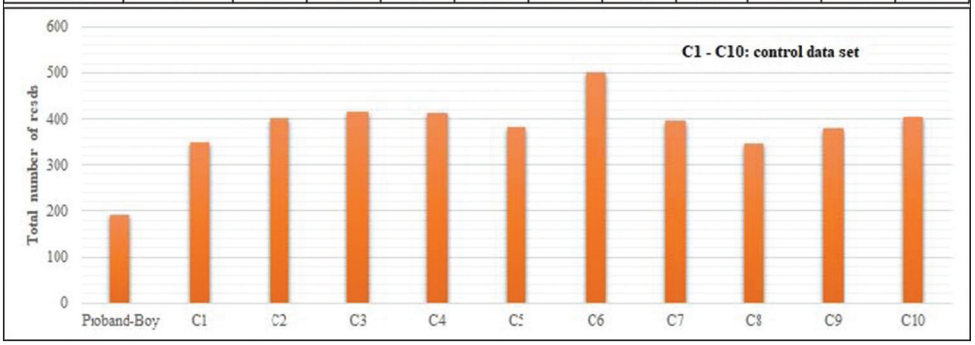
D. Database Information

Variants	Database reports						Predictions (scores)					
	1000 genomes	gnomAD	dbSNP	Indian Inhouse database	HGMD	ClinVar	SIFT	Polyphen-2	Mutation Taster2	M-CAP	CADD	InterVar
FUCA1:c.795G>C:p.Trp265Cys	NP	NP	NP	NP	NP	NP	Damaging	Probably Damaging	Disease causing	Possibly Pathogenic	31	Likely pathogenic

*NP – Not present

Supplementary Fig. 1. Data statistics and variant information tables. (**A**) Data quality and alignment statistics. (**B**) Variant filtering strategy using population databases and prediction scores (minor allele frequency≤0.01 cut-off). (**C**) Interpretation of the variant identified in *FUCA1* gene. (**D**) Information available in various databases about the *FUCA1*: c.795G>C:p.Trp265Cys variant, and the prediction scores calculated in various prediction tools.

FUCA1 -EXONS	Proband-Boy	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
exon1	1224	1092	1422	1218	256	243	1140	165	460	383	546
exon2	260	291	270	303	366	281	540	269	500	626	170
exon3	187	179	138	224	215	175	362	197	272	231	248
exon4	255	327	253	206	202	113	234	169	246	203	341
exon5	192	350	402	415	413	383	502	396	346	379	403
exon6	436	416	347	427	300	259	458	237	372	281	342
exon7	215	175	165	197	213	196	348	190	268	211	195
exon8	508	489	415	535	343	265	387	292	452	405	342



Supplementary Fig. 2. Table represents the read count distribution in all 8 exons of *FUCA1* gene, where exon 5 shows significant low count (highlighted) in proband boy of our sample set for whom WES data are available, C1-C10 shows 10 control samples data of WES sequences using same kit. Plot showing distribution of reads in exon 5 for the proband boy and control (C1-C10). WES, whole exome sequencing.

Quantitative real-time PCR (qPCR) analysis

Quantitative real-time PCR (qPCR) was performed with the Power SYBR Green PCR Master Mix (Takara Bio Inc., Tokyo, Japan) on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. *FUCA1* amplification was done using primers F: 5′- GGGGTCAGAACTGTTCCTGT-3′ and R: 5′- CAATGCCATGTCACGACGAT-3′. *GAPDH* was used as reference gene and amplified using primers F: 5′-TGCCTTCTTGCCTCTTGTCT-3′ and R: 5′-GGCTCACCATGTAGCACTCA-3′. The experiment was repeated twice with each single reaction in triplicate for all the four family members. Assays used DNA at 5 ng/µl final concentration and a no-template control for each primer set in triplicate. Relative gene dosage evaluated by $2^{-\Delta\Delta Ct}$ method calculating the difference in cycle number (ΔCt).

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