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PLoS one

Identification of Novel Mutations in *HEXA* Gene in Children Affected with Tay Sachs Disease from India

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Abstract

Tay Sachs disease (TSD) is a neurodegenerative disorder due to β-hexosaminidase A deficiency caused by mutations in the *HEXA* gene. The mutations leading to Tay Sachs disease in India are yet unknown. We aimed to determine mutations leading to TSD in India by complete sequencing of the *HEXA* gene. The clinical inclusion criteria included neuroregression, seizures, exaggerated startle reflex, macrocephaly, cherry red spot on fundus examination and spasticity. Neuroimaging criteria included thalamic hyperdensities on CT scan/T1W images of MRI of the brain. Biochemical criteria included deficiency of hexosaminidase A (less than 2% of total hexosaminidase activity for infantile patients). Total leukocyte hexosaminidase activity was assayed by 4-methylumbelliferyl-N-acetyl-β-D-glucosamine lysis and hexosaminidase A activity was assayed by heat inactivation method and 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulphate lysis method. The exons and exon-intron boundaries of the *HEXA* gene were bidirectionally sequenced using an automated sequencer. Mutations were confirmed in parents and looked up in public databases. *In silico* analysis for mutations was carried out using SIFT, Polyphen2, MutationT@ster and Accelrys Discovery Studio softwares. Fifteen families were included in the study. We identified six novel missense mutations, c.340 G>A (p.E114K), c.964 G>A (p.D322N), c.964 G>T (p.D322Y), c.1178C>G (p.R393P) and c.1385A>T (p.E462V), c.1432 G>A (p.G478R) and two previously reported mutations. c.1277_1278insTATC and c.508C>T (p.R170W). The mutation p.E462V was found in six unrelated families from Gujarat indicating a founder effect. A previously known splice site mutation c.805+1 G>C and another intronic mutation c.672+30 T>G of unknown significance were also identified. Mutations could not be identified in one family. We conclude that TSD patients from Gujarat should be screened for the common mutation p.E462V.

Citation: Mistri M, Tamhankar PM, Sheth F, Sanghavi D, Kondurkar P, et al. (2012) Identification of Novel Mutations in *HEXA* Gene in Children Affected with Tay Sachs Disease from India. PLoS ONE 7(6): e39122. doi:10.1371/journal.pone.0039122

Editor: Markus Schuelke, Charité Universitätsmedizin Berlin, NeuroCure Clinical Research Center, Germany

Received October 8, 2011; **Accepted** May 16, 2012; **Published** June 18, 2012

Funding: The work at FRIGE was supported by grant from Indian council of medical research (ICMR), project no BMS: 54/2010. The work at Genetic Research Center, NIRRH (National Institute for Research in Reproductive Health) was supported by intramural funds. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Tay Sachs disease (TSD) (MIM# 272800) is an autosomal recessive neurodegenerative disorder due to β-hexosaminidase A deficiency caused by mutation in the *HEXA* gene (MIM* 606869) encoding the α subunit of hexosaminidase A, a lysosomal enzyme composed of α and β polypeptides [1]. The clinical picture ranges from the acute infantile form rapidly leading to death to progressive later onset form compatible with a longer survival. Clinical features include neuroregression, generalized hypotonia, exaggerated startle response and cherry-red spot seen on fundus examination. Affected patients have deficient enzyme activity of Hexoasaminidase A (Hex A) in leukocytes or plasma [2]. The human *HEXA* gene is located on chromosome 15 q23-q24 with 14 exons. Nearly 130 mutations have been reported so far in the *HEXA* gene to cause TSD and its variants, including single base substitutions, small deletion, duplications and insertions splicing alterations, complex gene rearrangement and partial large duplications (http://www.hgmd.cf.ac.uk/). Most of these mutations are private mutations and have been present in a single or few families. Only few mutations have been commonly found in particular ethnicities or geographically isolated populations. In the Ashkenazi Jews, 94 to 98% patients are caused by one of the three common mutations c.1277_1278insTATC, c.1421+1 G>C and c.805 G>A (p.G269S) [3–5]. Among the non-Ashkenazi TSD patients the mutations pattern is completely different. A 7.6 kb deletion which includes the entire exon 1 and parts of the flanking sequence, is the major mutation causing TSD in the French Canadian population [4]. The mutation c.571–1 G>T accounts for 80% of mutant alleles among Japanese patients with TSD [1]. The c.1277_1278insTATC and the c.805 G>A(p.G269S) mutations are also commonly found in non-Ashkenazi Jewish populations, along with an intron 9 splice site mutation (c.1073+1 G>A) and the 7.6 kb French Canadian deletion. About 35% of non-Jewish individuals carry one of the two pseudodeficiency alleles; c.739C>T (p.R247W) and c.745C>T (p.R249W), which are not associated with neurological manifestations, since

Table 1. Clinical, biochemical and molecular details of the Indian patients with Tay Sachs disease.

S no	Age at diagnosis	Native State	Consan-guinity	Hex-A activity (MUGS) (nmol/hr/mg) = (x)	Total Hex activity (MUG) = (y)	Hex A % = (x/y) X 100	Genotypes (nucleotide level)	Genotypes (protein level)
1	11 months	Gujarat	No	4.5	ND	-	c.1385A>T/c.1385A>T	p.E462V/p.E462V
2	1.5 Yrs	Gujarat	No	4.8	1827.6	0.2	c.1385A>T/c.1385A>T	p.E462V/p.E462V
3	1 Yrs	Gujarat	No	5.9	2208.9	0.26	c.1385A>T/c.1385A>T	p.E462V/p.E462V
4	1.5 Yrs	Gujarat	No	5.1	2009	0.25	c.1385A>T/c.1385A>T	p.E462V/p.E462V
5	1.5 Yrs	Gujarat	No	5.8	2288	0.25	c.1385A>T/c.1385A>T	p.E462V/p.E462V
6	11 months	Gujarat	No	0.0 Father -60% (HLC) Mother –53% (HLC)	2131.0 731.7 528.3	0.0 - -	c.1385A>T/c.1385A>T	p.E462V/p.E462V
7	2.5 Yrs	Uttar Pradesh	No	4.08	1453.0	0.28	c.964 G>A/c.964 G>A	p.D322N/p.D322N
8*	15 months	Uttar Pradesh	No	0.0	716.2	0.0	Father and mother - [c.964 G>T/−]	p.D322Y/−
9	10 months	Maharashtra	No	23.8	1840.2	1.29	c. 340 G>A/c.340 G>A	p.E114K/p.E114K
10	1 Yrs	Maharashtra	No	7.2	2100	0.3	c.672+30T>G/c.1432 G>A	Undetermined/p.G478R
11	18 months	Tamil Nadu	Yes	17.2	2054.5	0.83	c.1178 G>C/c.1178 G>C	p.R393P/p.R393P
12*	1 Yrs	Andhra Pradesh	Yes	Father- 59.3% (HLC) Mother - 59.1% (HLC)	ND	-	Father -[c.805+1 G>C/−] Mother - [c.805+1 G>C/−]	-
13	1.5 Yrs	Iraq	Yes	18.4	1776.9	1.03	c.508C>T/c.508C>T	p.R170W/p.R170W
14	1 Yrs	Karnataka	Yes	1.5	1660	0.09	Not found	-
15	16 months	Gujarat	No	2.1	ND	_-	Father and mother - [c.1277_1278insTATC/-−]	

*DNA of index case is not available, HLC-heat labile activity in carrier parents, ND – not done.

Normal total hexosaminidase values using MUG substrate in our controls –703 to 1785 nmol/hr/mg protein, normal hexosaminidase A levels – (62 to 77%); normal MUGS activity 80 to 390 nmol/hr/mg.

doi:10.1371/journal.pone.0039122.t001

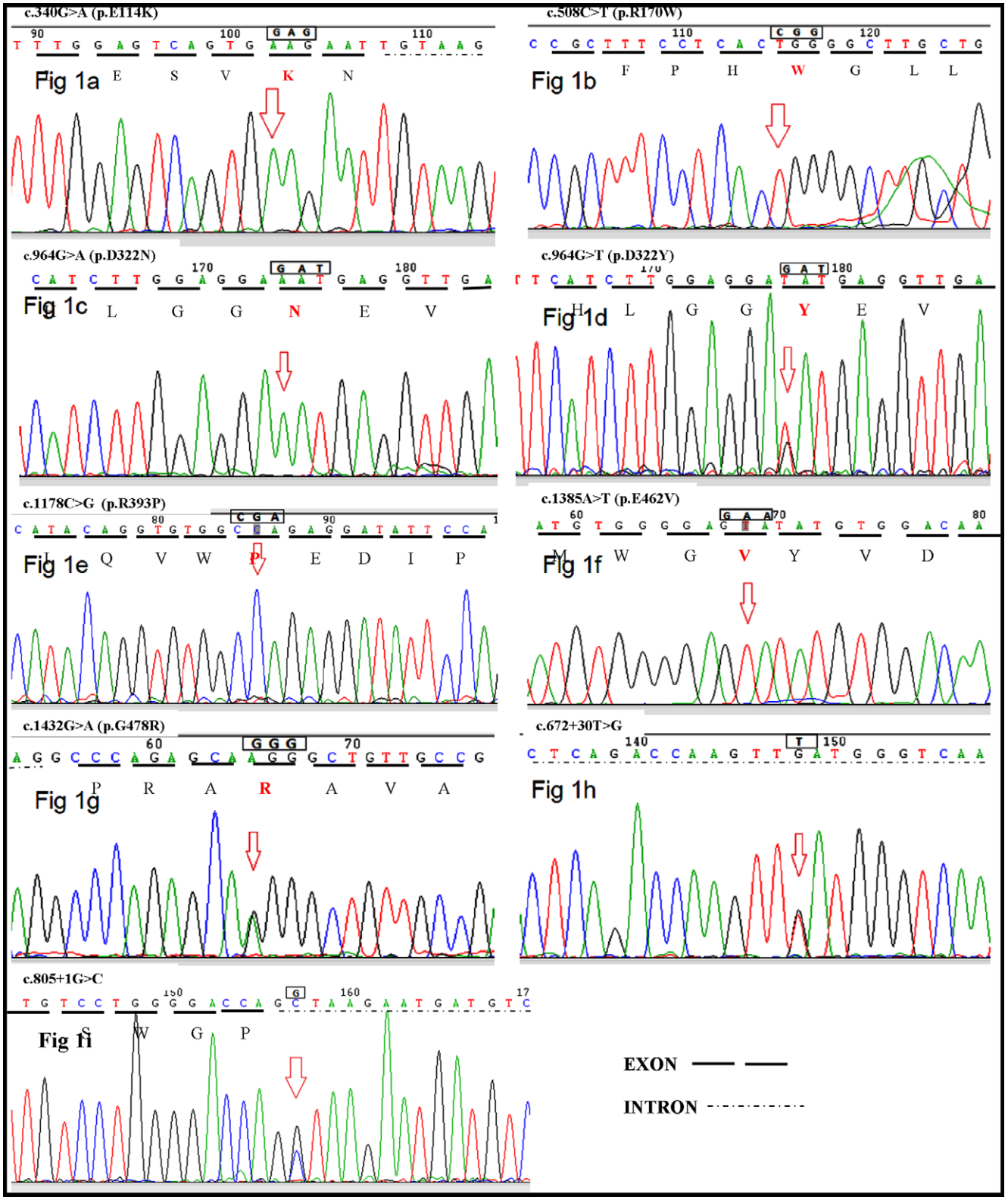

Figure 1. (a) – (e): Sequence Chromatogram of mutations Fig. 1a: c. 340 G>A (p.E114K)(homozygous); Fig. 1b: c.508C>T (p.R170W)(homozygous); Fig. 1c: c.964 G>A (p.D322N)(homozygous); Fig. 1d: c.964 G>T (p.D322Y)(heterozygous); Fig. 1e: c.1178 G>C (p.R393P) (homozygous); Fig. 1f: c.1385A>T (p.E462V) (homozygous); Fig. 1g: c.1432 G>A (p.G478R)(heterozygous); Fig. 1h: c.672+30T>G (heterozygous); Fig. 1e: c.805+1 G>C.
doi:10.1371/journal.pone.0039122.g001

their presence causes the reduction of Hex A activity only towards the artificial substrate but not to the natural GM2 ganglioside [6]. The mutations responsible for TSD in Indian patients are hitherto unpublished.

Results

Fifteen families were included in the study. Consanguinity in parents was present in 4/15 (26.7%) families. The mean age at presentation was 16.6 months (+/−5.4). All patients had seizures, neuroregression, exaggerated startle reflex, cherry red spot on fundoscopy, axial hypotonia, increased peripheral limb tone and brisk deep tendon reflexes. None of the patients had hepatosplenomegaly. Neuroimaging in form of computed tomography (CT scan) or magnetic resonance imaging (MRI) of the brain of the proband was available in 10/13 patients and showed characteristic findings of putaminal hyperintensity and thalamic hypointensity in CT scan or T2 weighted images of MRI of the brain. There were no white matter abnormalities. Significant deficiency of Hex A activity was observed in the leukocytes of all thirteen patients and only carrier detection (% Hex A) could be performed in two families where the proband was not alive (Table 1).

The DNA of fourteen such affected TSD families did not have the common mutations c.1277_1278insTATC, c.1421+1 G>C, c.805 G>A (p.G269S), 7.6 kb deletion or the two pseudodeficiency mutations c.739C>T (p.R247W) and c.745C>T (p.R249W). The common mutation c.1277_1278insTATC was detected by screening in one family and was confirmed by sequencing. Complete sequencing analysis revealed nine different mutations in thirteen families and could not identify any mutation in one family. We identified six novel deleterious missense mutations, c.340 G>A, c.964 G>A, c.964 G>T, c.1178C>G and c.1385A>T, c.1432 G>A, that resulted in amino acid changes p.E114K, p.D322N, p.D322Y, p.R393P, p.E462V, p.G478R [refer to Figure 1(a)–(d)]. We also identified known pathogenic mutations c.508C>T (p.R170W), c.805+1 G>C and another intronic variant c.672+30T>G of undetermined significance (refer to Table 1). The novel mutations were not found in 100 control individuals. The missense mutations were not found in the 1000 Genome Project. The intronic variant c.672+30T>G (rs117160567) was found in 1000 Genome Project (Minor Allele Frequency/Minor Allele Count: C = 0.09/20). The mutation c.1385A>T (p.E462V) was found in the homozygous state in six acute infantile TSD patients belonging to unrelated families with common ethnicity (from Gujarat) indicating a founder effect. Haplotype analysis was performed by sequencing the introns 1, 5, 12, 13 and a 417 basepair region 3′ to HEXA gene which contained several polymorphic markers, in all TSD patients and 30 ethnic controls. Table 2 reveals the haplotypes unique to the

Table 2. Haplotype analysis of founder mutation p.E462V in Gujarati patients with Tay Sachs disease.

3′ to HEXA gene(a)	Al	Intron 13 (b)	Al	Intron 12 (c)	Al	Intron 5 (d)	Al	Intron 1 (e)	Al
rs35949555	T	rs12904378	C	rs74020947	G	rs191809305	A	rs191094610	A
rs11629508	C	rs113387077	A	rs57733983	T	rs188570040	T	rs188410016	C
rs76075374	C	rs2912217	G	rs189856670	A	rs60920713	C	rs186683578	A
rs60288568	G	rs190224431	G	rs140288703	G	rs59427837	C	rs184065715	T
rs112626309	G	rs145393752	T	rs185764548	C	rs12910617	C	rs78629973	C
rs3087652	T	rs112806142	G	rs111680766	A	rs12593333	A	rs4470105	C
		rs890313	G	rs147324677	C	rs12592727	T	rs78278321	G
		rs111827252	C	rs113941121	A	rs4776594	G	rs76530364	T
		rs75015614	T	rs34085965	A			rs75756977	C
		rs62022857	C	rs2303448	C			rs75981720	C
		rs118002327	C					rs76941148	C
		rs2912218	T					rs80039124	T
		rs113665670	CTCT					rs78970750	A
		rs149948017	T					rs76950885	C
		rs4777502	C					rs74738827	A
		rs147502219	T					rs80238386	G
		rs140091006	G					rs77154656	C
		rs145012038	C					rs78097627	C
		rs149092488	G					rs77511366	A
								rs74325922	G
								rs74787391	C

(Al = allele).
Region (a) = 417 bp region 3′ to HEXA gene (chromosome 15 plus strand: 72635626 to 72636042); region (b) = 405 bp region in intron 13 of HEXA gene (chromosome 15 plus strand: 72637019 to 72637423; and 567 bp region in intron 13 of HEXA gene (chromosome 15 plus strand: 72637428 to 72637994; region (c) = 489 bp region in intron 12 of HEXA gene (chromosome 15 plus strand:72638306 to 72638794); region (d) = 424 bp region in intron 5 of HEXA gene (chromosome 15 plus strand: 72639456 to 72639879); region (e) = 405 bp region in intron 1 of HEXA gene (chromosome 15 plus strand: 72656546 to 72656950 and 461 bp region in intron 1 of HEXA gene chromosome 15 plus strand: 72659130 to 72659590). The chromosome.
coordinates are as per Human Genome Assembly Feb 2009 (GRCh37/hg19).
doi:10.1371/journal.pone.0039122.t002

Table 3. Details of *HEXA* missense mutations detected in infantile TSD patients and *in silico* analysis.

Exon no	Codon no.	Codon change	Amino acid change	Mutation T@ ster score	SIFT Score	Polyphen2 Score (sensitivity, specificity)	RMSD between native and mutant structure	Potential Energy after minimization (Kcal/mol) (native structure energy −25289.27)	Amino acid change
2	114	GAG – AAG	Glu114Lys	3.30 (DC)	0.00 (IT)	0.998 (0.16, 0.99) (PD)	0.134 (P)	−25045.67	Acidic to basic
5	170	CGG – TGG	Arg170Trp	2.75 (DC)	0.00 (IT)	1.00 (0, 1) (PD)	0.177 (DC)	−25025.01	Basic to nonpolar
8	322	GAT – AAT	Asp322Asn	0.63 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.176 (DC)	−25599.05	Acidic to Uncharged polar
8	322	GAT- TAT	Asp322Tyr	4.36 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.182 (DC)	−25476.57	Non- cyclic to cyclic
11	393	CGA - CCA	Arg393Pro	2.81 (P)	0.10 (T)	0.611 (0.8, 0.82) (PD)	0.387 (DC)	−24852.34	Basic to nonpolar
12	462	GAA - GTA	Glu462Val	3.3 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.178 (DC)	−25043.90	Acidic to nonpolar
13	478	GGG- AGG	Gly478Arg	3.41 (DC)	0.06 (T)	0.878 (0.69, 0.89) (PD)	0.453 (DC)	−24950.66	Nonpolar to Basic

DC = Disease causing, P = Polymorphism, IT = Intolerant, T = Tolerant, PD = Probably damaging RMSD = root mean square deviation.
The MutationT@sterscore is taken from an amino acid substitution matrix (Grantham Matrix [3]) which takes into account the physico-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid. Scores may range from 0.0 to 6.0.
The SIFT score is the normalized probability that the amino acid change is tolerated and ranges from 0 to 1. The amino acid substitution is predicted damaging is the score is $< = 0.05$, and tolerated if the score is >0.05.
The Polyphen2 score is the Naïve Bayes posterior probability that this mutation is damaging and thus ranges from 0 to 1.
doi:10.1371/journal.pone.0039122.t003

founder mutation and not found in other patients and 30 ethnic controls. The founder mutation was screened in 500 individuals using ARMS–PCR (amplification refractory mutation system-polymerase chain reaction) and two individuals were found to be carrier for this mutation leading to an allele frequency of 1/500. The ARMS method used the primers.

5′-CCAGGTTTGTGTTGTCCACATATT-3′ (mutation specific primer) and 5′-AGTTACCCCACCATCACCAGACTG-3′ (common forward primer) in a PCR reaction to show presence of p.E462V allele (123 base pair PCR product) and the primers 5′-CCAGGTTTGTGTTGTCCACATATA-3′ (wild type specific primer) and the common forward primer in another PCR reaction to show presence of wild type allele (123 base pair PCR product). Carriers were confirmed by sequencing.

The Sorting Tolerant from Intolerant (SIFT) index (available at http://sift.jcvi.org/), Polyphen2 scores (available at http://genetics.bwh.harvard.edu/pph2/) and MutationT@ster (available at http://www.mutationtaster.org/) scores for the non-synonymous single nucleotide substitutions are given in Table 3. [7–9] The predictions of the SIFT, Polyphen2 and MutationT@ster algorithms were in concordance with the observed pathogenicity in case of mutations p.E114K, p.R170W, p.D322N, p.D322Y, and p.E462V. The RMSD (Root mean square deviation) values for the modeled mutants were significant for pathogenicity for all missense mutations except p.E114K (refer to Table 3 and Figure 2). This mutation was determined pathogenic by SIFT and Polyphen analysis. The docking studies reveal that the residues αAsp322, αTyr421, αArg178, αGlu462 play a crucial role in the binding and orientation of GalNAc (refer to Figure 3 and Tables 3, 4 and 5).

The intronic mutation c.672+30T>G was evaluated for pathogenicity using MutationT@ster. The mutation was predicted to lead to gain of aberrant donor splice site at position c.672+35 (score 0.5). However no functional studies could be carried out further since the family was not available later for further testing. It was not found in 100 controls with normal Hex A values. Another intronic mutation c.805+1 G>C disrupts the normal donor splice site of the intron 7. This was found in a consanguineous carrier couple from Andhra Pradesh with history of a previous child being affected with Tay Sachs disease (refer to Table 1).

Discussion

The clinical and neuroimaging features of infantile Tay Sachs disease seen in our patients were consistent with the defined phenotype. The results of enzyme activity measurements (Hex A expressed as percentage of total Hex activity) varied from 0% to 1.29%. This is consistent with previous observations that infantile TSD patients have values ranging from 0 to 2% [10]. Recent studies in patients from India have revealed similar values in infantile TSD patients [11]. Our study indicates that the known deleterious common mutations c.1278insTATC, c.1421+1 G>C, c.805 G>A (p.G269S), 7.6 kb deletion and the two pseudodeficiency mutations c.739C>T (R247W) and c.745C>T (R249W) are not common in Indian TSD patients.

We found three novel deleterious mutations (p.D322N, p.D322Y, p.E462V) occurring at the functionally active site of the alpha subunit of hexosaminidase A. The mutation p.R393X has been previously identified in infantile TSD patients from Persia, Turkey and Iraq populations [12–14]. The mutation p.E114K is proximal to the N-glycosylation site α-N115.

We were especially interested in analyzing the c.1385 T>A (p.E462V) mutation because it was identified in six unrelated families from one ethnic group but, to our knowledge, has never

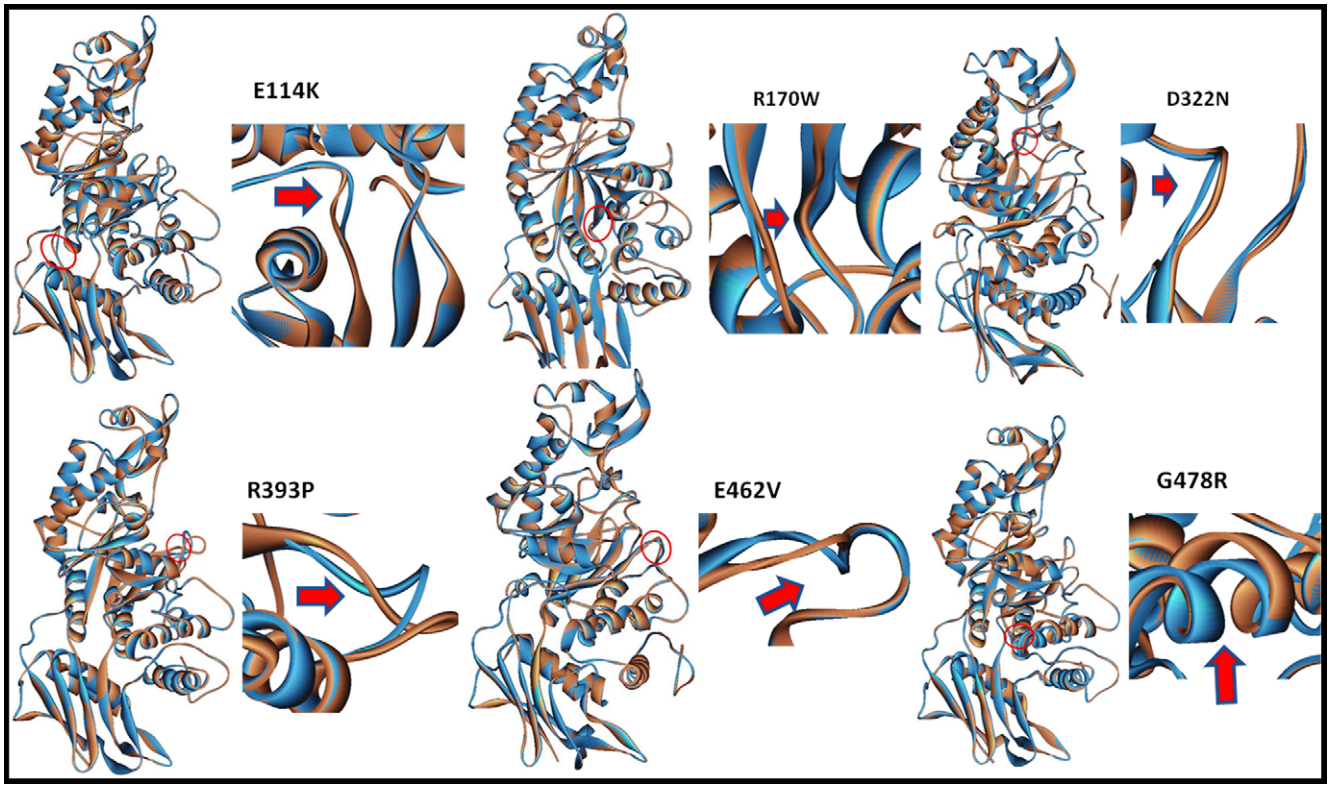

Figure 2. Superimposed native structures (blue) and mutant structure (brown) of the α subunit produced using Accelrys Discovery Studio software from top left clockwise: a) mutation p.E114K causes conformational change, b) p.R170W disrupts the beta sheet c) p.D322N affects the active catalytic site, d) p.R393P causes conformational change, e) p.E462V affects the active site and the dimerization of alpha-beta subunits, f) p.G478R disrupts the alpha helix.
doi:10.1371/journal.pone.0039122.g002

been identified in TSD patients of other ethnic origin. The sequence alignment of Hex A from various species (refer to Figure 4) reveal that the residue E462 is highly conserved. The RMSD of the modeled mutant is also significantly higher as compared to the wild-type protein (refer to Table 1 and Figure 2). This mutation was present in homozygous state in all six patients exhibiting infantile TSD. This is in accordance with previous observations that missense mutations responsible for infantile TSD were generally located in a functionally importance region, such as the active site and the dimer interface [15].

The mutation c.508C>T (p.R170W) has been previously reported in different ethnic groups [Japanese, French-Canadian (Estree region, Quebec) and Italian patients] [16–18]. The side chain of R170 in domain II forms a hydrogen bond with the E141 in domain I. The substitution of R with W with a bulkier side chain has a large effect on the interface of domains I and II. This would destabilize the domain interface and cause degradation of the α-subunit [16], [19].

The intronic mutation c.672+30T>G has been previously reported in an obligate carrier individual detected on biochemical screening. The mutation has been reported to be benign in this paper as it was found in combination with another disease causing mutation in an obligate carrier [20]. The National Center for Biotechnology Information (NCBI) single nucleotide polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/SNP/index.html) revealed that the minor allele count of this polymorphism (rs117160567) is minimal (C = 0.0103/13). However this allele has not been reported in homozygous state till date. Thus, the pathogenic potential of this mutation remains to be explored by functional analysis. The presence of another *HEXA* mutation in this patient in form of large heterozygous deletion or mutation in regions not analyzed like deep intronic and promoter cannot be ruled out.

The splice site mutation c.805+1 G>C has been reported previously from Portuguese patients [21]. Another substitution at the same nucleotide c.805+1 G>A is common in French-Canadian patients from the Quebec region in Canada [22]. Both mutations are known to be associated with the infantile form of TSD.

In one of our patients, no mutation could be identified. Complete gene sequencing strategy is likely to miss large heterozygous deletions or gene rearrangements. Southern blot analysis or multiplex ligase dependent probe amplification (MLPA) based methods to detect these changes were not available to us.

A large number of gene mutations causing TSD have been previously reported in the *HEXA* mutation database http://www.hexdb.mcgill.ca/. Among them, mutations resulting in gross alterations in the Hex α-subunit sequence are generally found in the severe infantile form. However, missense mutations causing amino acid substitutions have been found in both the infantile and late onset phenotypes [23]. Mahuran et al, (1999) reported that the detrimental effect of most mutations is on overall folding and/or transport of the protein rather than on the functional sites [24]. Some of the mutations identified in our series affect crucial active residues or cause significant structural aberrations leading to the infantile form of the disease.

Conclusion

The mutations responsible for Tay Sachs disease in Indian population are unique. Patients originating from Gujarat state

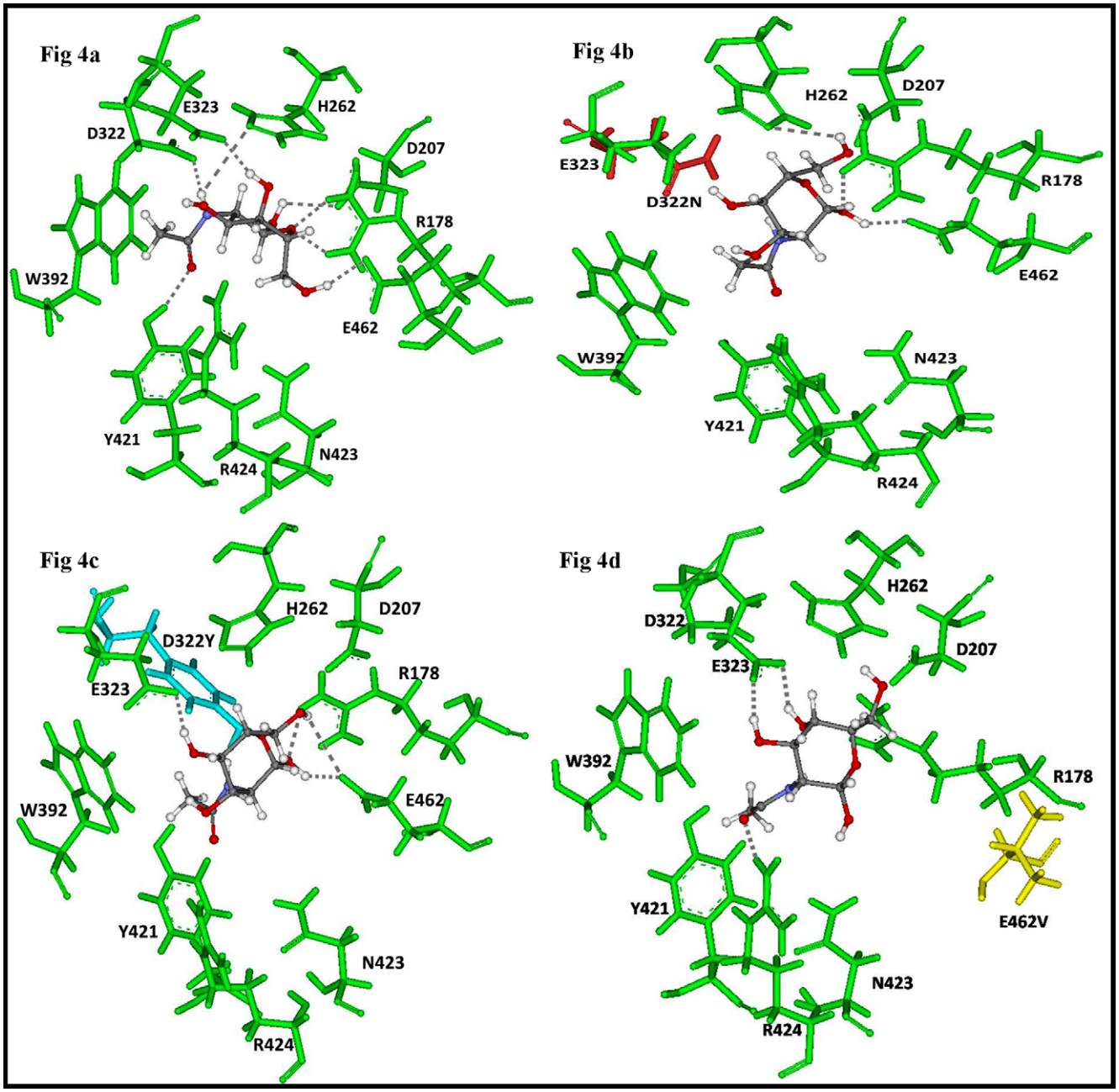

Figure 3. Stereoscopic view of the docking experiments. Green sticks indicate C^{α} trace of amino acids involved in the active site and CPK (Corey-Pauling-Koltun) coloring scheme represents ligand GalNAc (N acetyl galactosamine) portion of GM2 ganglioside. Fig 4a: Wild type (αHex-A-GalNAc complex). Fig 4b: Mutant (αD322N-GalNAc), Red shows mutation p.D322N, Fig 4c: Mutant (αD322Y-GalNAc), Cyan shows mutation p.D322Y, Fig. 4d: Mutant (α E462V-GalNAc), yellow shows mutation p.E462Y.
doi:10.1371/journal.pone.0039122.g003

could be screened for the founder mutation p.E462V. Except for this mutation, the rest of the mutations were non-recurrent. However, screening for the above mutations would be recommended for cost effective testing of TSD patients.

Methods

Ethics Statement

The study protocol was approved by Ethics Committee of Foundation for Research in Genetics and Endocrinology, Ahmedabad, Gujarat (Reg No- E/13237).

Study Subjects

Clinical details were noted in a case record form and an informed written consent was obtained from each family. The clinical criteria for inclusion in this study was history of neuroregression, exaggerated startle reflex, cherry red spots on fundus examination and relative macrocephaly. Neuroimaging criteria included presence of gray matter disease shown by hyperintensity in basal ganglia and/or hypointensity in thalamus in T2 weighted images of MRI of the brain. Five ml peripheral blood was collected from the patients for leukocyte enzyme assay and DNA extraction. Leukocyte hexosaminidase A activity was

Table 4. Hydrogen bond interaction for the wild type and Mutant complexes.

Complex	Protein (Residue:Atom)	Ligand Atom
Wild Type-GalNAc	ARG178:HH12	O2
	ARG178:HH22	O2
	TYR421:HH	O6
	TRP460:HE1	O6
	ASP207:OD1	H26
	GLU323:OE1	H28
	GLU462:OE2	H29
	HIS262:NE2	H30
	ASP322:OD2	H20
αD322N-GalNAc	ARG178:HH22	O1
	GLU462:OE2	H26
	HIS262:NE2	H29
αD322Y-GalNAc	ARG178:HH22	O1
	GLU462:OE2	H26
	GLU323:OE2	H28
	GLU462:OE2	H29
αE462V-GalNAc	ARG424:HH21	O6
	GLU323:OE2	H27
	GLU323:OE1	H28

doi:10.1371/journal.pone.0039122.t004

determined by manual fluorimetric enzyme assay. Total hexosaminidase (Hex) was measured from the hydrolysis of the synthetic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine (MUG), which releases fluorescent 4-methylumbelliferone when acted upon by β-hexosaminidase. Hexosaminidase B (Hex B) was determined as the activity left after samples were heated for three hours at 50°C. This procedure led to loss of Hex A which is heat-labile but not of hexosaminidase B (Hex B) or intermediate isoenzyme. Therefore, Hex A activity was obtained after subtracting Hex B activity from total Hex activity. Hex A was also assayed with the more specific sulphated substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulphate (MUGS) [25]. Hex A activity (obtained from MUGS lysis) was expressed as percentage of total Hex activity (obtained from MUG lysis).

Screening Procedure for Common Mutations

Genomic DNA was extracted by the standard salting out method. Primary screening of the common mutations

Table 5. Ligand Binding Energy Details for wildtype and mutant α subunits.

Complex	Binding Energy (kcal/mol)	Complex
Wild Type-GalNAc	−115.94	Wild Type-GalNAc
αD322N-GalNAc	−38.87	αD322N-GalNAc
αD322Y-GalNAc	−52.22	αD322Y-GalNAc
αE462V-GalNAc	−80.88	αE462V-GalNAc

doi:10.1371/journal.pone.0039122.t005

c.1277_1278insTATC, c.1421+1 G>C, 7.6 kb deletion, the pseudo-deficiency alleles (p.R247W and p.R249W) was performed by amplification refractory mutation system – polymerase chain reaction (ARMS PCR) using normal and mutant primers as a reverse primers and common forward primers as previously described [26], [27]. PCR amplification was performed for each mutation separately in a 15 ul final reaction volume containing 500 ng genomic DNA, 10× Cetus buffer, 2.0 mM dNTPs, 1 U Taq polymerase, 4–6 pmol of each primers and 1.5 µl 10× Cetus buffer. The thermocycling conditions were 10 min at 94°C, followed by 25–32 cycles of amplification consisting 30 s–60 s at 94°C, 30 s–60 s at 55–63°C and 30 s –3 min at 72°C, and a final elongation for 10 min at 72°C and PCR product were run on 2% agarose and visualized under ultraviolet transilluminator.

Molecular Analysis of *HEXA* Gene

The exonic and intronic flanking sequence of the *HEXA* gene were PCR amplified in 14 fragments using previously described primers [28]. DNA amplification was performed for each fragment in a 10 µl final volume containing 100 ng genomic DNA, 1 mM dNTPs, 10 pmol of each primer, 1 U Taq polymerase and 1 µl 10 X PCR buffer. Thirty cycles of amplification were used, each consisting 1 min denaturation at 94°C, 45 s annealing at 60–65°C suitable for each exons and 45 s extension at 72°C in a thermal cycler. Final extension time was 10 minutes. Negative control PCR tubes contained all of the above ingredients except DNA. PCR products were then electrophoresed in 2% agarose along with the appropriate negative controls and a 100 base-pair DNA ladder. Products that passed this quality check were purified by treatment with Exo-SAP-IT ™ (USB Corporation, OH, USA) and then sequenced using BigDye Terminator v3.1 and capillary electrophoresis was performed using an automated sequencer ABI-3130 (Applied Biosystems, CA, USA). Mutations were described according to mutation nomenclature, considering nucleotide +1 the A of the first ATG translation intitiation codon. Nucleotide numbers are derived from cDNA HEXA sequence (RefSeq cDNA NM_000520.4). Putative mutations were confirmed in two separate PCR products from the patient's DNA. Heterozygosity for these mutations was confirmed in the parents. The mutations identified were then looked up in public databases like The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html) and McGill University database (http://www.hexdb.mcgill.ca). Novel variants were also ruled out as polymorphism by sequencing the corresponding exons/introns in 100 normal unrelated individuals.

In *Silico* Analysis

Prediction of functional effects of non-synonymous single nucleotide substitutions (nsSNPs) was done using softwares SIFT (Sorting Intolerant From Tolerant) (available at http://sift.jcvi.org/), Polyphen2 (Polymorphism Phenotyping v2) (available at http://genetics.bwh.harvard.edu/pph2/) and MutationT@ster (available at http://www.mutationtaster.org/). [7–9] HumVar-trained prediction model of Polyphen2 was used for distinguishing mutations with drastic effects from all the remaining human variation, including abundant mildly deleterious alleles. Evolutionary conservation of the amino acid residues of Hex A was analyzed using ClustalW program available online at http://www.uniprot.org/help/sequence-alignments.

Structural Studies

Protein Preparation. The mutants (αD322N, αD322Y, αE462V)were built using build mutant protocol of Accelrys Discovery studio 2.0 using the crystallographic structure of Hex A

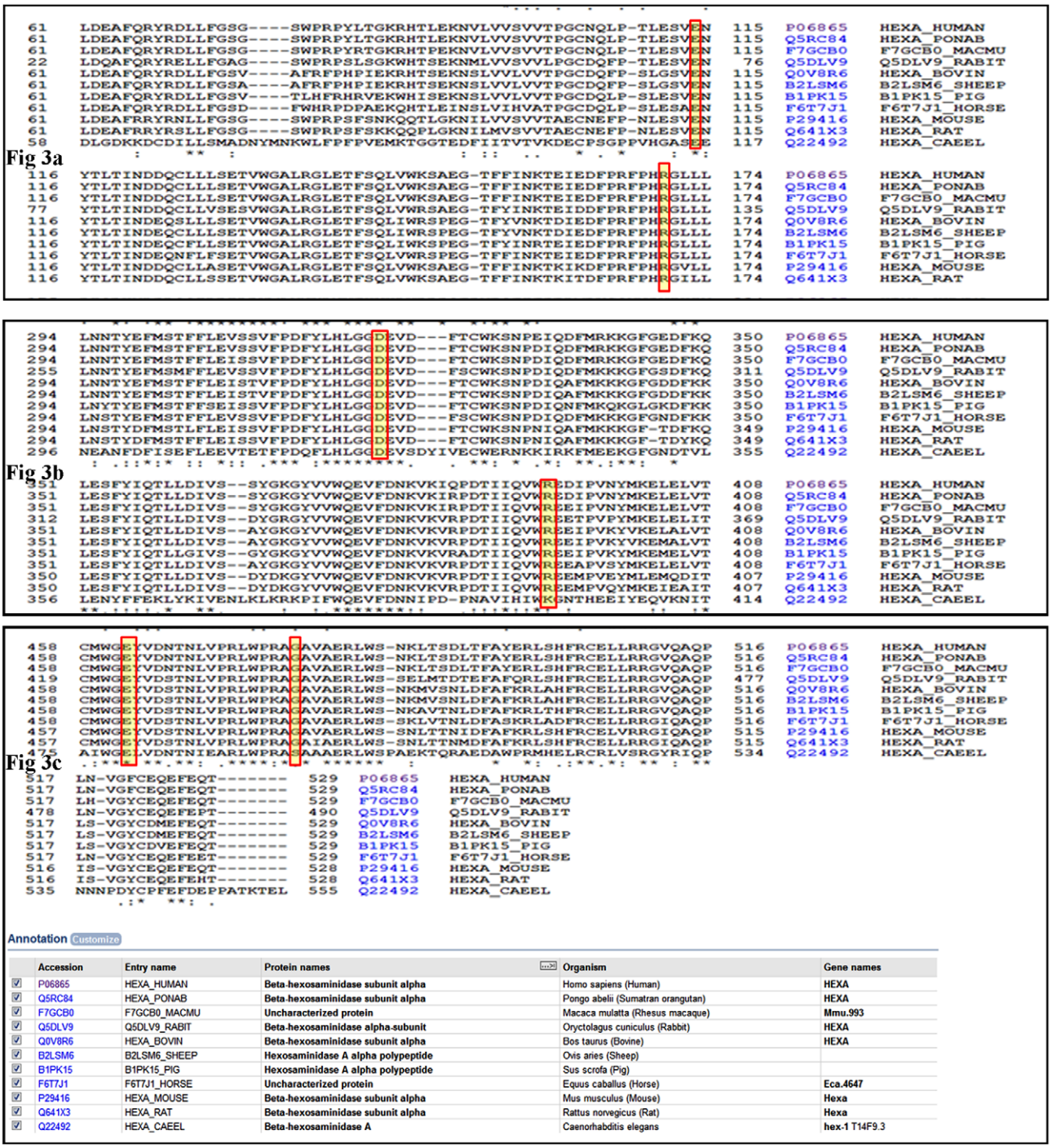

Figure 4. Multiple protein sequence alignment using ClustalW shows evolutionary conservation of amino acid residues. Fig. 3a: αE114 and αR170; Fig. 3b: αD322 and αR393; Fig. 3c: αE462 and αG478.
doi:10.1371/journal.pone.0039122.g004

(PDB ID: 2 GJX) as the template. The wild type protein and mutants were energy minimized using CHARMm forcefield and 200 cycles of steepest descent algorithm.

Ligand Preparation. The structural coordinates of GM2 ganglioside was retrieved from the PubChem database (CID 9898635). The GalNAc (N-acetyl galactosamine) portion of the GM2 ganglioside was considered for docking on the active site of Hex A as reported previously [29]. It was prepared using the Prepare Ligands protocol of Accelrys Discovery studio 2.0.

Molecular Docking. Structural analysis revealed that residues R178, D207, H262, E323, D322, W373, W392, Y421, N423, R424 and E462 constitute the active site of αHex-A. This information was used to define the binding site and docking studies were performed using the LigandFit protocol of Accelrys Discovery studio 2.0. The potential energy and Root Mean

Square Deviations (RMSD) of the mutant structures with respect to the wild-type structure were calculated. RMSD values more than 0.15 were considered as significant structural perturbations that could have functional implications for the protein [15].

Acknowledgments

We would like to thank the Director of NIRRH, Dr. Sanjiva Kholkute for his support. We also thank Rochelle Tixeira for technical assistance. We thank the families of patients with Tay Sachs disease for their participation in this research.

Author Contributions

Conceived and designed the experiments: PMT JS SIT SG. Performed the experiments: PMT MM FS DS PK SP SIT SG. Analyzed the data: MM PMT JS SIT SP. Contributed reagents/materials/analysis tools: PMT JS FS SG. Wrote the paper: PMT MM JS FS.

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JIMD Reports
DOI 10.1007/8904_2013_244

RESEARCH REPORT

Burden of Lysosomal Storage Disorders in India: Experience of 387 Affected Children from a Single Diagnostic Facility

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Received: 04 April 2013 / Revised: 16 May 2013 / Accepted: 26 May 2013 / Published online: 13 July 2013

Abstract Lysosomal storage disorders (LSDs) are considered to be a rare metabolic disease for the national health forum, clinicians, and scientists. This study aimed to know the prevalence of different LSDs, their geographical variation, and burden on the society. It included 1,110 children from January 2002 to December 2012, having coarse facial features, hepatomegaly or hepatosplenomegaly, skeletal dysplasia, neuroregression, leukodystrophy, developmental delay, cerebral-cerebellar atrophy, and abnormal ophthalmic findings. All subjects were screened for I-cell disease, glycolipid storage disorders (Niemann-Pick disease A/B, Gaucher), and mucopolysaccharide disorders followed by confirmatory lysosomal enzymes study from leucocytes and/or fibroblasts. Niemann-Pick disease-C (NPC) was confirmed by fibroblasts study using filipin stain. Various storage disorders were detected in 387 children (34.8 %) with highest prevalence of glycolipid storage disorders in 48 %, followed by mucopolysaccharide disorders in 22 % and defective sulfatide degradation in 14 % of the children. Less common defects were glycogen degradation defect and protein degradation defect in 5 % each, lysosomal trafficking protein defect in 4 %, and transport defect in 3 % of the patients. This study demonstrates higher incidence of Gaucher disease (16 %) followed by GM2 gangliosidosis that includes Tay-Sachs disease (10 %) and Sandhoff disease (7.8 %) and mucopolysaccharide disorders among all LSDs. Nearly 30 % of the affected children were born to consanguineous parents and this was higher (72 %) in children with Batten disease. Our study also demonstrates two common mutations c.1277_1278insTATC in 14.28 % (4/28) and c.964G>T (p.D322Y) in 10.7 % (3/28) for Tay-Sachs disease in addition to the earlier reported c.1385A>T (p.E462V) mutation in 21.42 % (6/28).

Communicated by: Verena Peters

Competing interests: None declared

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Introduction

Lysosomal storage disorders (LSDs) are the consequence of an abnormal storage of undigested cellular debris, proteins, fats, carbohydrates, and nucleic acids within the cell (Parkinson-Lawrence et al. 2010). They occur due to mutations in the genes that encode for lysosomal hydrolases, resulting in an attenuated enzyme activity and/or their transport to the lysosomes (Saftig and Klumperman 2009; Vellodi 2005). To date, nearly 50 such enzyme deficiencies are responsible for around 40 known storage diseases that have been identified (Vellodi 2005; Futerman and van Meer 2004).

Individually, these disorders are rare but collectively they are as common as other metabolic disorders with a reported incidence of 1:7,700 in Australia (Meikle et al. 1999). A newborn screening program in Taiwan identified a surprisingly high frequency of Taiwanese males with Fabry disease (~ 1 in 1,250). Hwu et al. (2009) identified IVS4+919G>A cryptic mutation in 86 % of the Fabry patients having a late-onset cardiac phenotype. Similarly, Gaucher disease is also one of the most common genetic disorders in Ashkenazi Jews, with a frequency of 1 in 855 live births (Staretz-Chacham et al. 2009). In the Ashkenazi Jews, 94–98 % patients with Tay-Sachs disease have three common mutations: c.1277_1278 insTATC, c.1421+1G>C, and c.805 G>A (p.G269S) (Myerowitz and Costigan 1988; Kaback et al. 1993), while a 7.6 kb deletion is the major mutation causing Tay-Sachs disease in the French Canadian population (Myerowitz and Hogikyan 1986).

A few Indian studies (Sheth et al. 2004; Verma 2000; Nalini and Christopher 2004; Verma et al. 2012) have partly addressed the incidence of LSDs in India along with mutation detection for Tay-Sachs disease (Mistri et al. 2012) and metachromatic leukodystrophy (MLD) (Shukla et al. 2011). Considering the large population and a high frequency of consanguineous marriages, the incidence of LSDs is likely to be higher in India. Hence, this study has been planned to know the burden of LSDs in high-risk group of children and identify common mutation for Tay-Sachs disease in the country, which is more commonly seen in schedule caste (SC) community of Gujarat (Mistri et al. 2012).

Material and Methods

This work is a prospective randomized study of 1,110 children referred from various Indian states and a couple of neighboring countries (Sri Lanka and Afghanistan) from January 2002 to December 2012. It comprises of 938 (84.50 %) children from western, 121 (10.9 %) from southern, 30 (2.7 %) from northern, 1 (0.09 %) each from eastern and central parts of India, 18 (1.62 %) from Sri Lanka, and 1 (0.09 %) from Afghanistan in the age range of 1 day to 16 years. This study includes 738 male and 372 female children. The most common phenotypes for the referral were coarse facial features, hepatomegaly or hepatosplenomegaly, skeletal dysplasia, neurological involvement including developmental delay or neuroregression, spasticity, seizures, leukodystrophy, cerebral-cerebellar atrophy, and abnormal ophthalmic findings such as cherry red spot, vision loss, and corneal clouding. An institutional ethics committee approval and patient informed consent was obtained to carry out this study.

Random urine and peripheral whole blood samples were collected in all the cases and were initially processed for the screening test followed by confirmatory enzyme study. The screening study was carried out from urine samples for glycosaminoglycans (GAGs) [both quantitative and qualitative] (Dembure and Roesel 1991; Hopwood and Harrison 1982) and plasma was analyzed for chitotriosidase activity (Aerts et al. 2008; Sheth et al. 2010) and mucolipidosis II/III [ML II/III] screening (Sheth et al. 2012). Leukocytes were isolated from whole blood and were processed by standard protocol for enzyme assay using 4-MU fluorometric assay or PNCS spectrophotometric synthetic substrate as outlined by Shapria et al. (1989); Filocamo and Morrone (2011); and Sheth et al. (2004). Niemann-Pick disease-C (NPC) study was carried out on cultured skin fibroblasts in lipid-deficient medium using filipin stain (Kruth and Vaughan 1980; Sheth et al. 2008).

The mutation analysis was carried out in 28 children with enzymatically confirmed diagnosis of Tay-Sachs disease. Genomic DNA was extracted by the standard salting out method (Miller et al. 1988) and investigated for common mutations c.1277_1278insTATC, c.1421+1 G>C, 7.6 kb deletion, the two pseudo-deficiency alleles c.739C>T (p.R247W) and c.745C>T (p.R249W) of *HEXA* gene followed by exon and intron flanking sequencing for known, rare, and private mutations in all the cases. Prediction of functional effects of non-synonymous single nucleotide substitutions (nsSNPs) was done using softwares SIFT (Sorting Intolerant From Tolerant) and Polyphen2 (Polymorphism Phenotyping v2).

Results

Out of 1,110 children, 387 (34.8 %) were found to be affected with different storage disorders (Table 1 and Fig. 1). Of these, 115 children (29.6 %) were born to consanguineous parents. Glycolipid storage disorders were the most commonly diagnosed LSDs (48 %) in this study population followed by mucopolysaccharide (MPS)

Table 1 Clinical and biochemical study of 387 children affected with different lysosomal storage disorders in India

Disease name/(enzyme name)/[substrate name]	Phenotype observed	Age at diagnosis	Number of cases investigated	Number of affected cases confirmed by enzymatic analysis	Enzyme activity detected in affected individuals (nmol/h/mg protein) Enzyme activity from leukocytes observed in normal individuals (nmol/h/mg protein)	Genotype analysis
Defects in degradation of glycolipids						
Gaucher disease (β-glucosidase) [4-MU-β-D-glucopyranoside]	*Type 1* – chronic non-neuropathic: severe hepatosplenomegaly, chronic anemia, and thrombocytopenia; Gaucher cells present in bone marrow	6 M–13 Y	425	60 (15.5 %)	0.0–4.6 8.0–32.0	Not yet carried out
	Type 3 – subacute neuropathic: degenerative of central nervous system, peripheral symptoms similar to type 1	10 M–3 Y	425	02 (0.51 %)	0.0–4.0 8.0–32.0	Not yet carried out
Tay-Sachs disease (β-hexosaminidase A) [4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulfate (MUGS)]	Neuroregression after 6 months of age, cherry red spot, vision loss, seizures, hypotonia, exaggerated startle response, progressive deafness, delayed mile stone, microcephaly, cerebral atrophy, partial optic atrophy	10 M–3 Y	465	39 (10 %)	0.0–23.4 80.4–410.0	*Earlier reported mutations*[a] c.340G>A (E114K) [exon 2]/c.340G>A (E114K) [exon 2] ($n = 1$) c.964G>A (D322N) [exon 8]/c.964G>A (D322N) [exon 8] ($n = 1$) c.964G>A (D322N) [exon 8]/ c.1277_1278insTATC [exon 11] ($n = 1$) c.964 G>T (D322Y) [exon 8]/c.964G>T (D322Y) [exon 8] ($n = 3$) c.1178C>G (R393P) [exon 11]/c.1178 C>G (R393P) [exon 11] ($n = 1$) c.1385A>T (E462V) [exon 12]/c.1385 A>T (E462V) [exon 12] ($n = 6$) c.1432G>A (G478R) [exon 13]/ c.672+30T>G [inton 6] ($n = 1$) c.1277_1278insTATC [exon 11]/ c.1277_1278insTATC [exon 11] ($n = 3$) c.508C>T (R170W) [exon 5]/c.508C>T (p.R170W) [exon 5] ($n = 1$) c.805+1 G>C [intron 7]/c.805+1G>C [intron 7] ($n = 2$)

(continued)

Table 1 (continued)

Disease name/(enzyme name)/[substrate name]	Phenotype observed	Age at diagnosis	Number of cases investigated	Number of affected cases confirmed by enzymatic analysis	Enzyme activity detected in affected individuals (nmol/h/mg protein) Enzyme activity from leukocytes observed in normal individuals (nmol/h/mg protein)	Genotype analysis
						Novel mutations c.788C>T (T263I)/c.788C>T (T263I) [exon 7] ($n = 1$) c.1121A>C (Q374P)/c.1121 A>C (Q374P) [exon10] ($n = 1$) c.1421G>A (W474X)/c.1420 G>A (W474X) [exon 12] ($n = 1$) c.1454G>A (W485X)/c.1454G>A (W485X) [exon 13] ($n = 2$) c.898-905delTTCATGAG/c.899-906delTTCATGAG [exon 8] ($n = 1$)
Sandhoff disease (β-hexosaminidase T) [4-MU-N-Ac-β-D-glucosaminide]	Neuroregression after 6 months of age, cherry red spot, vision loss, seizures, hypotonia, exaggerated startle response, progressive deafness, delayed mile stone, peripheral neuropathy, and organomegaly	7 M–3.5 Y	489	30 (7.75 %)	33.0–382.0 723.4–2700.0	Not yet carried out
Niemann-Pick disease A/B (Sphingomyelinase) [Hexadecarbonylamino-p-nitro-phenyl phosphoryl choline]	NPD A – neuropathicEarly life progressive loss of motor and intellectual capacity, hepatosplenomegaly, cherry red spot in macula, pneumonia, foamy histiocytes in bone marrow	5 M–2.3 Y	374	07 (1.8 %)	0.1–0.35 0.77–2.33	Not yet carried out
	NPD B – non-neuropathicLate onset as compared to type A, hepatosplenomegaly, cherry red spot in macula, foamy histiocytes in bone marrow	3–13 Y	374	17 (4.4 %)	0.25–0.46 0.77–2.33	Not yet carried out
GM1 gangliosidosis (β-galactosidase) [4-MU-β-D-galactopyranoside]	Cherry red spot, neuroregression, mild to moderate dysostosis multiplex, coarse facies, hypotonia, delayed mile stone, hepatosplenomegaly, mongolian spot on back, MRI shows diffuse dysmyelination of white matter with bilaterally symmetric thalamic signal change	7 M–4 Y	1,045	30 (7.75 %)	0.0–15.3 79.6–480.0	Not yet carried out

Defects in degradation of mucopolysaccharides

Hurler syndrome (MPS I) (α-iduronidase) [4-MU-α-L-iduronide]	*MPS IH – Hurler syndrome* Progressive mental and physical disabilities, corneal clouding, coarse facies, dysostosis multiplex, stiff joints, organomegaly, hernia	7 D–2.1 Y	355	12 (3.1 %)	0.0–12 32.0–105.5	Not yet carried out
	MPS IHS-Hurler-Scheie/Scheie syndrome Progressive physical disabilities, corneal clouding, coarse facies, dysostosis multiplex, stiff joints, glaucoma, hernia	3–9 Y	355	09 (2.32 %)	2.1–12 32.0–105.5	Not yet carried out
Hunter syndrome (MPS II) (α-iduronidase-sulfatase) [4-MU-α-L-iduronide-2-sulfate]	Severe mental retardation, dysostosis multiplex, coarse facies, organomegaly	6 M–7 Y	30	09 (2.32 %)	0.0–9.3[b] 600–1616[b]	Not yet carried out
Sanfilippo syndrome A (MPS IIIA) (Heparan sulfamidase) [4-MU-α-D-sulfoglucosaminide]	Aggressive behavior, mental retardation, joint stiffness, hirsutism	2.5–9 Y	32	08 (2.0 %)	0.01–0.24[c] 1.3–6.8[c]	Not yet carried out
Sanfilippo syndrome B (MPS IIIB) (N-acetyl-α glucosaminidase) [4-MU-N-Ac-α-glucosaminide]	Similar to MPS type IIIA	5–6.5 Y	128	02 (0.51 %)	0.6–4.7[b] 300–600[b]	Not yet carried out
Morquio syndrome A (MPS IVA) (β-galactosidase-6-sulfate-sulfatase) [4-MU-β-galactose-6-sulfate]	Skeletal abnormality, short stature, short neck, prominent lower ribs, odontoid anomalies, coarse facies	1.6–9 Y	54	18 (4.65 %)	4.0–5.2 14.0–32.0[c]	Not yet carried out
Morquio syndrome B (MPS IVB) (β-galactosidase) [4-MU-β-D-galactopyranoside]	Mild skeletal dysplasia, short stature, corneal clouding, coarse facies	6 M–10 Y	1,045	04 (1.03 %)	2.2–24.2 79.6–480.0	Not yet carried out
Maroteaux-Lamy syndrome (MPS VI) (Arylsulfatase B) [P-nitro-catechol-sulfate]	Severe dysostosis multiplex, corneal clouding, coarse facies, cardiomyopathy	1 M–10 Y	389	18 (4.65 %)	0.0–0.25 0.65–8.5	Not yet carried out
Sly syndrome (MPS VII) (β-glucuronidase) [4 MU-β-D-glucuronide]	Mental retardation, coarse facies, organomegaly, corneal clouding, mild skeletal abnormality	5 M–12 Y	372	05 (1.29 %)	0.0–27.8 80–400.0	Not yet carried out

(continued)

Table 1 (continued)

Disease name/(enzyme name)/[substrate name]	Phenotype observed	Age at diagnosis	Number of cases investigated	Number of affected cases confirmed by enzymatic analysis	Enzyme activity detected in affected individuals (nmol/h/mg protein) Enzyme activity from leukocytes observed in normal individuals (nmol/h/mg protein)	Genotype analysis
Defects in degradation of sulfatides						
Metachromatic leukodystrophy (MLD) (Arylsulfatase A) [P-nitro-catechol-sulfate]	Peripheral neuropathy, absence of deep tendon reflexes, slowly progressive dementia, gait disturbance, convulsion, behavior changes. MRI shows diffuse symmetric abnormalities of periventricular myelin with hyperintensities on T_2-weighted images	1 M–16 Y	483	38 (9.82 %)	0.0–0.3 0.6–4.5	Not yet carried out
Krabbe disease (β-galactocerebrosidse) [6-hexadeconylamino-4-methylumbelliferyl-β-D-Galactopyranoside]	Progressive psychomotor retardation, convulsion, spasticity, absence of deep tendon reflexes, irritability, hyperthermia; progressive, diffuse, and symmetric cerebral atrophy is observed in neuroimaging	1 M–15 Y	85	17 (4.39 %)	0.0–5.50[c] 15.2–114.9[c]	Not yet carried out
Defects in degradation of glycoproteins						
Neuronal ceroid lipofuscinosis I (NCL I) (Palmitoyl protein thioesterase)[4-MU-6-sulfo-palmitoyl-β-D-glucoside]	Early onset psychomotor impairment, vision loss, GTC convulsion, dementia, spasticity; MRI shows variable cerebral atrophy and thalamic hypointensity in the white matter and basal ganglia	6 M –2 Y	39	05 (1.28 %)	3.0–9.3 25.5–215	Not yet carried out
Neuronal ceroid lipofuscinosis II (NCL II) (Tripeptidyl peptidase 1) [Alanyl-alanyl-phenylalanyl-7-amido-4-methylcoumarin (AAF-AMC)]	Late onset psychomotor impairment, vision loss, GTC convulsion, dementia, spasticity; MRI shows progressive cerebral and cerebellar atrophy with normal basal ganglia and thalami	2–16 Y	84	13 (3.08 %)	4.3–22.5 32.8–233	Not yet carried out

Defects in degradation of glycogen						
Pompe disease (α-1-4-glucosidase) [4-MU-α-D-glucopyranoside]	Recurrent hypoglycemic seizures, cardiomegaly and hypertrophic cardiomyopathy, delayed milestone, muscle weakness, noisy breathing, respiratory distress, mild hepatosplenomegaly, gait disturbance	1 D –13 Y	384	19 (4.9 %)	0.0–0.19[d] > 0.25–0.63[d]	Not yet carried out
Defects is lysosomal transporters						
Sialic acid storage disorder [N-acetyl-neuraminic acid (NANA)]	Regression motor and physical milestone, cherry red spot, coarse facial features, hepatosplenomegaly, gum hypertrophy, inguinal hernia	1 M –9 Y	45	07 (1.80 %)	Free NANA[e] 7.83 (0–6 M) 4.4–17.22 (7–24 M) 4.75 (61–120 M) Total NANA[e] 8.48 (0–6 M) 3.9–20.0 (7–24 M) 5.75 (0–6 M) Free NANA[e] 0.42–1.70 (0–6 M) 0.26–1.39 (−24 M) 0.1–0.38 (61–120 M) Total NANA[e] 0.92–3.0 (0–6 M) 0.55–2.31 (7–24 M) 0.2–0.64 (61–120 M)	Not yet carried out
Galactosialidosis (β-galactosidase) [4-MU-β-D-galactopyranoside] and [N-acetyl-neuraminic acid total (Total NANA)]	Regression of motor milestone, seizures, coarse facies, organomegaly, mongolian spot, mild skeletal abnormality	3 M –2 Y	09	03 (0.77 %)	β-galactosidase 79.6–480.0 Free NANA[e] 0.6–0.7 (0–6 M) 0.51 (13–24 M) Total NANA[e] 3.5–8.25 (0–6 M) 2.8 (13–24 M) β-galactosidase 79.6–480.0 Free NANA[e] 0.42–1.70 (0–6 M) 0.31–0.79 (13–24 M) Total NANA[e] 0.92–3.0 (0–6 M) 0.55–1.47 (13–24 M)	Not yet carried out

(continued)

Table 1 (continued)

Disease name/(enzyme name)/[substrate name]	Phenotype observed	Age at diagnosis	Number of cases investigated	Number of affected cases confirmed by enzymatic analysis	Enzyme activity detected in affected individuals (nmol/h/mg protein) Enzyme activity from leukocytes observed in normal individuals (nmol/h/mg protein)	Genotype analysis
Defects in lysosomal of trafficking proteins						
Mucolipidosis II/III	Delayed milestone, coarse features, kyphosis, mild scoliosis, hypertonia, skin pigmentations, low-set ears, mild hepatosplenomegaly, mongolian spot, thick skin	7 M–6.5 Y	179	11 (1.78 %)	Aryl sulfatase-A (416.2–2828.0)[b] β-hexosaminidse T (66560–333300)[b] β-glucuronidase (18700–40546)[b] α-fucosidase (26500–32000)[b] Aryl sulfatase-A (28–85)[b] β-hexosaminidse T (12000–30149)[b] β-glucuronidase (420–2054)[b] α-fucosidase (334–1275)[b]	Not yet carried out
Niemann-Pick disease C[f]	Psychomotor symptoms occurring in early age, neonatal jaundice, organomegaly, hypertonic limbs, facial dyskinesia	1–6 Y	11	4 (1.03 %)	Depressed cholesterol esterification and abnormal filipin staining Normal cholesterol esterification and normal filipin staining	Not yet carried out

Y Years; *M* Months

[a] (Mistri et al. 2012)

[b] Enzyme activity was carried out in plasma and activity was expressed in nmol/h/ml plasma

[c] Enzyme activity was expressed in nmol/17 h/mg protein

[d] Enzyme activity is expressed by calculating ratio of with acarbose and without acarbose

[e] Enzyme activity was carried out in urine mmol/g creatinine

[f] NPD-C was carried out in skin fibroblasts using filipin staining

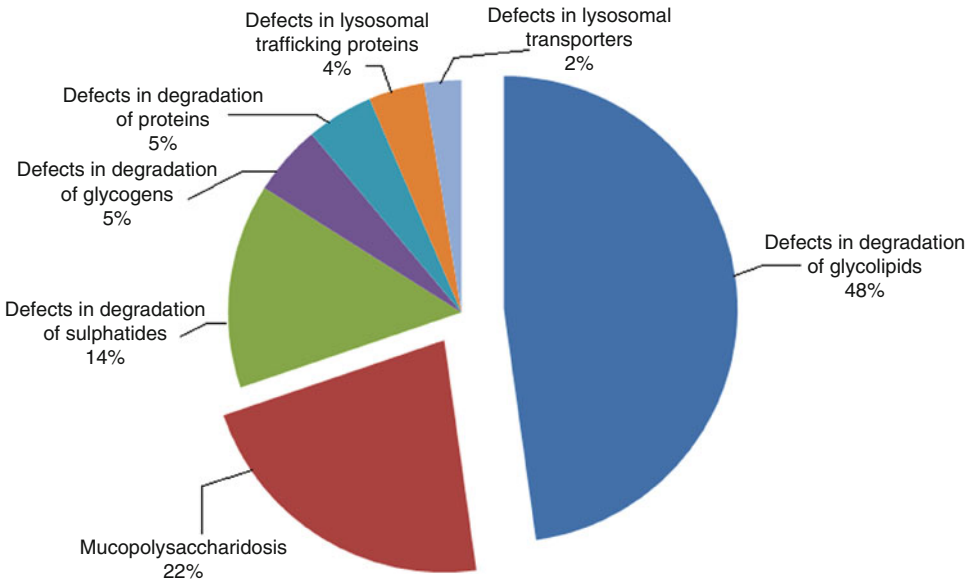

Fig. 1 Distribution of storage disorders in India

disorders (22 %) and sulfatide degradation defect (MLD and Krabbe disease) in 14 % of the patients. Glycogen storage disorder type II (Pompe disease), protein degradation defect (Batten disease), lysosomal trafficking protein defect (ML II/III and NPC), and lysosomal transporter defect (sialic acid storage disorder and galactosialidosis) were found to be comparatively less common (Fig. 1). Interestingly, all confirmed cases of Batten disease were from South India where consanguineous marriages were observed in 72 % of the cases.

Gaucher disease was one of the most common glycolipid storage disorders observed with a high frequency in Maharashtra 64.5 % (40/62) followed by GM2 gangliosidosis (Tay-Sachs disease and Sandhoff disease), which was more prevalent in Gujarat 49.7 % (34/69). Among MPS disorders, MPS I (Hurler, Hurler-Scheie, and Scheie syndrome) was the most common, followed by MPS IV (Morquio A and Morquio B), MPS VI (Maroteaux-Lamy syndrome), MPS II (Hunter syndrome), MPS III A and B (Sanfilippo syndrome A and B), and MPS VII (Sly syndrome). The remaining were sulfatide degradation defects with MLD and Krabbe disease, protein degradation defect with Batten disease (NCL I and II), lysosomal transporter defect with sialic acid storage disorder, and lysosomal trafficking protein deficiency with ML II/III and NPC (Table 1).

Mutation analysis was carried out for 28 children with confirmed diagnosis of Tay-Sachs disease. Common mutation c.1277_1278insTATC was detected in four cases and was confirmed by bidirectional sequencing. In the remaining 24 cases, exon sequence study revealed 14 different mutations, except one. This includes nine earlier reported mutations c.340G>A (p.E114K), c.1178C>G (p.R393P), c.1432G>A (p.G478R), c.508C>T (p.R170W) in one each, c.964G>A (p.D322N) in two, c.964G>T (p.D322Y) in three, and c.1385A>T (p.E462V) in six cases. Intron 7 showed variant c.805+1 G>C in two cases and intron 6 demonstrated c.672+30 T>G in one case. Additionally, two novel deleterious missense mutations c.788C>T (p.T263I) and c.1121A>C (p.Q374P) along with one small novel deletion c.898_905delTTCATGAG was detected in one case each. Two novel nonsense mutations c.1421G>A (p.W474X) and c.1454G>A (p.W485X) were observed in one and two cases, respectively. Pathological significance of novel missense mutations was confirmed using softwares SIFT and Polyphen2. Mutation spectrum of Tay-Sachs disease detected in various states of India is shown in Table 2, which also includes one case from Iran.

Discussion

This study demonstrates glycolipid storage disorders as one of the most common LSDs in India, similar to that observed in Portugal, Australia, and Czech Republic (Pinto et al. 2004; Meikle et al. 1999; Poupetova et al. 2010) with higher frequency of Gaucher disease (Fig. 2). The high prevalence of Gaucher disease (16 %) is in accordance with previously reported study (14.4 %) from northern India (Verma et al. 2012) and Mappila Muslims from southern India encompassing Kerala (Feroze et al. 1994).

GM2 gangliosidosis accounted for 10 % of Tay-Sachs and 7.8 % of Sandhoff patients, respectively. This was in accordance to the Portugal cohort especially for Tay-Sachs disease (13.8 %), while lower prevalence was

Table. 2 Regional distribution of mutation spectrum in Tay-Sachs disease

Disease condition	Gene involved	Mutation detection	Number of cases	Region – Country
Tay-Sachs disease	*HEXA*	c.1385 A>T (p.E462V)	6	Gujarat – India
		c.1277_1278insTATC (p.Y47IfsX5)	2	Gujarat – India
			2	Maharashtra – India
		c.964 G>T (p.D322Y)	2	Gujarat – India
			1	Uttar Pradesh – India
		c.964 G>A (p.D322N)	1	Gujarat – India
			1	Uttar Pradesh – India
		c.805+1G>C	1	Maharashtra – India
			1	Andhra Pradesh – India
		c.340G>A (p.E114K)	1	Maharashtra – India
		c.1432G>A (p.G478R)	1	Maharashtra – India
		c.672+30T>G	1	Maharashtra – India
		c.1178 C>G (p.R393P)	1	Tamil Nadu – India
		c.508C>T (p.R170W)	1	Iran
		c.788C>T (p.T263I)	1	Maharashtra – India
		c.1454G>A (p.W485X)	2	Maharashtra – India
		c.1121A>C (p.Q374P)	1	Maharashtra – India
		c.1421G>A (p.W474X)	1	Maharashtra – India
		c.898_905delTTCATGAG	1	Maharashtra – India

reported from Australia and Czech Republic (4.1 % and 2.5 %, respectively). High prevalence of GM2 gangliosidosis (Tay-Sachs disease and Sandhoff disease) has also been reported in children with neurological disorders from the southern region of India, where consanguinity is more common (Nalini and Christopher 2004). Higher incidence of Tay-Sachs in our study could be due to the presence of founder mutation in *HEXA* gene in the SC community of Gujarat (Mistri et al. 2012).

Mucopolysaccharidosis was the second most common LSD, which is in accordance with several other groups (Pinto et al. 2004; Meikle et al. 1999; Poupetova et al. 2010; Verma et al. 2012). Among these groups, MPS I was the most prevalent. This finding is similar to that observed by Verma et al. (2012). Although identification of MPS IV B in four cases is unique in our study, this could be either due to ethnic diversity or referral bias. A study from Czech Republic (Poupetova et al. 2010) reported only one case of MPS IV B out of 394 affected patients. Nonetheless, the prevalence of MPS IV A was found to be higher as compared to IV B which is in concordance with other reported studies (Pinto et al. 2004; Meikle et al. 1999; Poupetova et al. 2010). Occurrence of MPS III-A and III-B in this study is lower than that reported from Australia and Portugal (Pinto et al. 2004; Meikle et al. 1999) (Fig. 2). Milder clinical presentation, lack of clinical awareness, and diagnostic facility could be one of the reasons for underdiagnosis of MPS III disorders in this work.

In this study, the frequency of Pompe disease (GSD II) was higher (5 %) as compared to Czech Republic, Australia, and Portugal (Fig. 2). While Batten disease [Neuronal ceroid lipofuscinosis I and II (NCL I and II)] was observed in lower frequency (4 %) as compared to 7.5 % in Czech Republic, it was higher than that reported in Portugal (1.5 %). Interestingly, all cases of Batten disease in our study belonged to a single region of South India where higher incidence of consanguineous marriages is seen (72 %).

High carrier frequency of Fabry disease has been observed in Czech Republic and Taiwan, however, not a single case was detected in this study (Fig. 2) (Poupetova et al. 2010; Staretz-Chacham et al. 2009). This could be due to lack of awareness among clinicians involved in the cardiac and/or renal management of adults with Fabry disease. Unlike in Czech Republic, Australia, and Portugal (Meikle et al. 1999; Poupetova et al. 2010; Pinto et al. 2004), NPC was also the least observed lysosomal trafficking disorder. This could be a result of overlapping clinical phenotypes such as hepatosplenomegaly, neonatal jaundice, and psychiatric disturbances (Wenger et al. 2003) and limited investigational facilities for NPC.

In general, low prevalence of storage disorders like Fabry, NPC, and MPS III in our study is likely to be due to the lack of awareness among the clinicians and a dearth of enzyme diagnostic facilities in most parts of the country. In countries with higher incidences of aforementioned

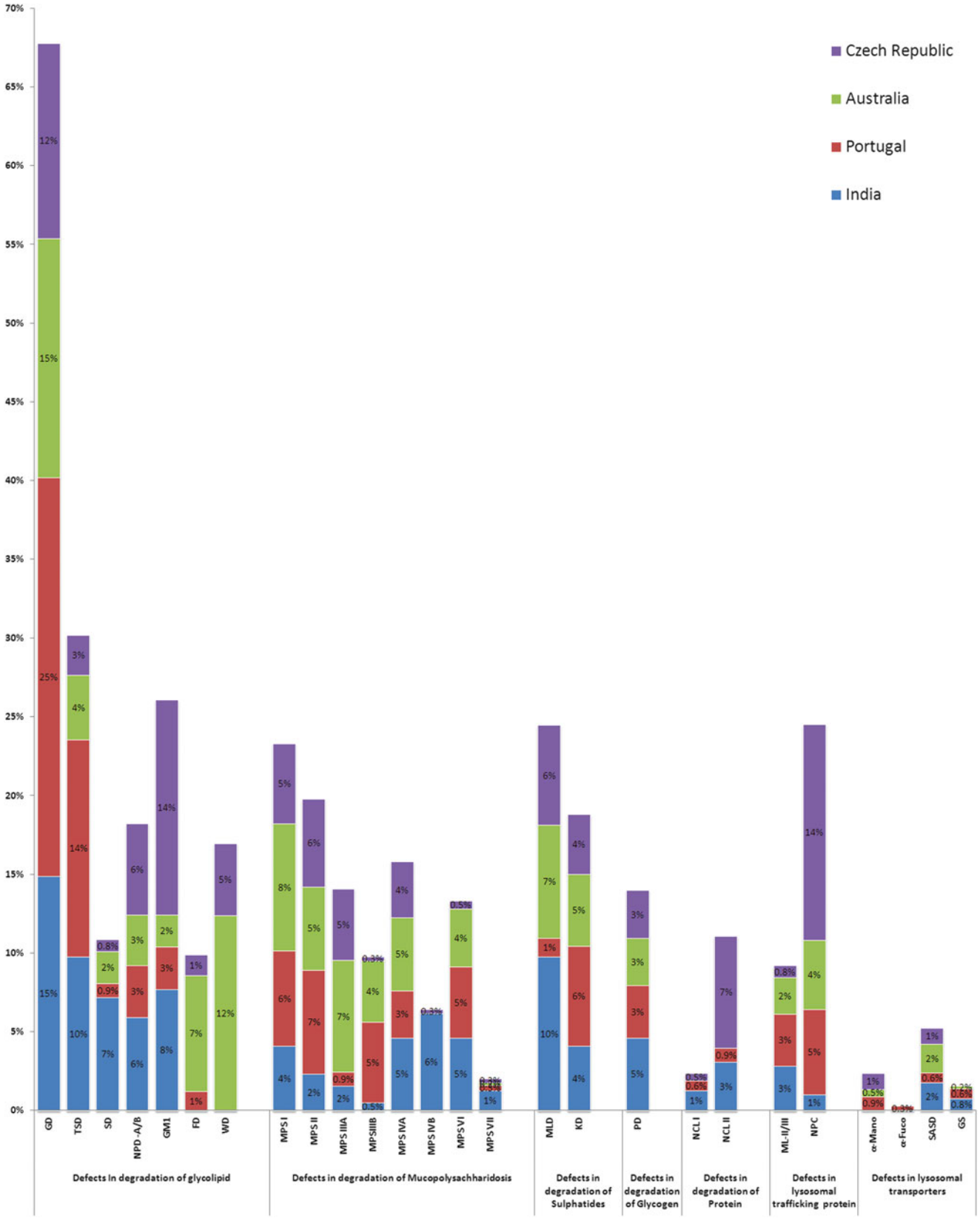

Fig. 2 Incidence of various LSDs in different countries

disorders, easy accessibility of specialized enzyme laboratory for confirming clinical diagnosis exists. Additionally, in these countries (Australia, Portugal, and Czech Republic) special working groups on LSDs increase awareness among the medical fraternity. Also patient support groups advocate the disease magnitude and support the affected families (Meikle et al. 1999; Pinto et al. 2004; Poupetova et al. 2010). In India, very recently Indian Council of Medical Research (ICMR) has set up a special task force on LSDs which will focus on the magnitude of these disorders in different parts of the country, increase awareness among the clinicians by organizing regional training program, and establish common mutation spectrum for different LSDs.

The ethnicity predilection is known to be associated with a specific lysosomal disease like Gaucher, Tay-Sachs, Niemann-Pick type A, and ML IV disease in Ashkenazi Jewish descendants (Marsden and Levy 2010); Salla disease and aspartylglucosaminuria in Finnish population; and Gaucher type III disease in people of Swedish descent (Marsden and Levy 2010). In this study, the prevalence of Gaucher disease was found to be higher in Maharashtra region, whereas GM2 gangliosidosis (Tay-Sachs) was more in Gujarat province.

Among all the LSDs, molecular analysis was carried out in 28 cases of Tay-Sachs disease by mutation identification in *HEXA* gene. Molecular study in 15 of these cases has been reported earlier with high prevalence of p.E462V mutation in a particular community (SC) of Gujarat (Mistri et al. 2012). In the remaining 13 cases, 6 aforementioned novel mutations have been observed in patients from Maharashtra region (Table 2) of the country that include two missense mutations in exon 7 and exon 10, two nonsense mutations in exon 12 and exon 13, and one small deletion (8 bp deletion) in exon 8. Deleterious effect of these mutations has been confirmed by SIFT and Polyphen2 software programs. The remaining seven patients have shown earlier reported mutations with c.1277_1278insTATC in three, p.D322Y in two, p.D322N and c.805+1G>C in one each. Thus, this study shows that p.E462V, p.D322Y, and c.1277_1278insTATC identify nearly 45 % (13/28) of the disease causing mutations in patients affected with Tay-Sachs disease from India.

Overall, the study demonstrates that glycolipid storage and MPS are the most common LSDs in India. By providing appropriate genetic counseling and offering prenatal diagnosis during subsequent pregnancies, the burden of these diseases could be reduced. Mutations p.E462V, p.D322Y, and c.1277_1278insTATC are possibly the commonest ones detected for Tay-Sachs disease in the Indian population and can be used as a part of common mutation screening program.

Acknowledgments We sincerely acknowledge the work of Meikle PJ, Pinto R, and Poupetova H and their colleagues from Australia, Portugal, and Czech Republic, respectively, whose publications were of immense help in the preparation of this manuscript. We are grateful to all the referring doctors and patients for their support without whose consent, this study could not have been possible and Indian Council of Medical Research (ICMR) for providing financial support to carry out this study.

Synopsis

This cross-sectional study provides an up-to-date description of the current scenario of different lysosomal storage disorders (LSDs) and its burden on the society and highlights the common mutation spectrum for Tay-Sachs disease in India.

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

JS designed the experiment and standardized the protocols. MM, NO, and CA were involved in processing of the samples. MJ provided the technical guidelines. RS, AB, KG, NN, CD, MK, and SM were involved in collection of the clinical details. MM, JS, and FS prepared the manuscript. All the authors read and approved the manuscript.

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J. Fetal Med. (March 2014) 1:17–24
DOI 10.1007/s40556-014-0001-3

ORIGINAL ARTICLE

Prenatal Diagnosis of Lysosomal Storage Disorders by Enzymes Study Using Chorionic Villus and Amniotic Fluid

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Received: 17 April 2014 / Accepted: 20 May 2014 / Published online: 3 July 2014

Abstract The reported prevalence of lysosomal storage disorder (LSD) is 1:5,000–7,000 live births and with the limited availability of therapeutic option; prenatal diagnosis (PD) remains the only preventable cure for storage disorders. One hundred forty pregnancies having confirmed diagnosis of LSDs in index case were selected for enzymes study during PD from uncultured and/or cultured chorionic villus (CV/CCV) and cultured amniotic fluid (CAF) cells. In seven pregnancies, molecular analysis was additionally carried out where mutation was known in an index case. Of 140 pregnancies, 60 (42.9 %) were diagnosed as affected, 13 (9.3 %) had an intermediate enzyme activity and 67 (47.8 %) had normal enzyme activity. Results were confirmed in 83 cases whereas 57 cases were lost from the follow-up. In one case, enzyme β-galactose-6 sulphate sulphatase specific for Morquio-A disorder [mucopolysaccharidosis-IVA (MPS-IVA)] had shown 30 % reduced activity in CV cells and the case was diagnosed as carrier for MPS-IVA while it delivered an affected child. Further molecular analysis in seven cases that included six with Tay-Sachs and one with Gaucher disease, confirmed the results obtained by enzymatic study during PD. PD of LSDs can be carried out by enzymes study from CV/CCV and CAF with an accuracy of molecular method. However, in cases of MPS and mucolipidosis, Amniotic fluid (AF) is preferred over CV/CCV. In addition, special care is needed while interpreting enzyme results encompassing carrier status and they need to be further evaluated by molecular studies.

Keywords Lysosomal storage disorders · Prenatal diagnosis · Enzyme assays · Chorionic villus sampling · Amniotic fluid studies

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Introduction

Prenatal diagnosis (PD) is one of the important approaches for the prevention of lysosomal storage disorders (LSDs) where treatment options are either beyond the financial reach of the family or are not available. Various methods have been employed in the PD of LSDs that include morphological study of uncultured chorionic villus (CV) cells for vacuoles [1], enzyme study from CV cells, cultured chorionic villus (CCV) and/or cultured amniotic fluid (CAF) cells [2], electrophoresis of AF for excretion pattern of various mucopolysaccharides (MPSs) [3], electroscopic ionization tandem mass spectrometry [4] and mutation analysis using DNA from CV and AF [5].

Molecular studies provide precise PD of the LSDs. However, mutation detection in an affected child is the

prerequisite [6], which is not available in most of the cases with storage disorders in developing countries due to the cost and absence of common mutation screening. Hence, lysosomal enzyme studies are important for PD by most of the centers where the diagnosis in the index case has been confirmed [1, 2, 7]. Very few reports are available from India where LSDs were detected during PD using lysosomal enzymes [8–10]. Nonetheless, considering the reported prevalence of the disease [11], the significant burden of various LSDs reported by Sheth et al. [12] from India and its limited therapeutic options, PD remains the only preventable measure for LSDs. The present study was carried out to evaluate the sensitivity and specificity of lysosomal enzyme assays for the PD of LSDs from CV/CCV and CAF cells.

Materials and Methods

A prospective study of 140 pregnancies from 2006 to 2013 was carried out from CV/CCV and/or CAF cells for the PD of LSDs by lysosomal enzyme where the diagnosis in the index case was confirmed. A written informed consent was obtained from the patients as per the Prenatal Diagnosis Act and counseling was provided to each family.

Prenatal sampling was done depending upon the stage of pregnancy, fetal position and maternal health. CV sampling and/or amniocentesis was carried out between 10–13 and 15–18 weeks of gestation, respectively as per the standard procedure. Enzyme analysis was carried out from CV cells in 16 cases, CV and CCV both in 19 cases, CCV alone in 19 whereas CAF cells were analyzed in 86 cases by the method described below. All prenatal diagnostic samples were simultaneously processed with sample matched positive and negative controls for the required enzymes.

The CV samples were collected in a sterile collection vial containing a medium to minimum quantity of 10–15 mg. After checking for maternal cell contamination, they were thoroughly washed with sterile phosphate buffered saline (PBS) followed by distilled water. CV samples were homogenized on ice. After repeated freezing and thawing, supernatant was used for direct enzyme study with protein concentration between 2 and 10 mg/mL [1].

After thorough examination and washing with PBS, CV samples were chopped and cultured in growth medium under 5 % CO_2 as per standard protocol [1, 2]. Once cell confluence was obtained, which usually takes 2 weeks time, they were harvested carefully and protein activity was determined. Final concentration of protein was kept 2–10 mg/mL and it was used for enzyme study.

About 15–20 mL of fluid was collected without maternal blood contamination. Following centrifugation, cells were cultured in two different growth mediums. After cell confluence was attained, the grown fibroblasts were processed for different lysosomal enzyme study after protein estimation [1, 2].

Enzyme activity was carried out using the synthetic substrate 4-methylumbelliferrone-fluorogenic substrate and *p*-nitrocatechol sulfate-spectrophotometric substrate [13]. As shown in Table 1, the present study included 140 patients for various LSDs depending upon the diagnosis in affected sibs. The enzyme activity was expressed as nmol/h/mg of protein except nmol/4 h/mg of protein for α-iduronate sulfatase and nmol/17 h/mg of protein for β-galactose-6-sulphate-sulfatase, heparan sulphamidase and β-galactocerebrosidase.

Genomic DNA was isolated from CV/CCV and CAF cells using salting out method [14]. Molecular analysis was carried out in seven pregnancies that included six with Tay-Sachs disease (*HEXA* gene) and one with Gaucher disease (*GBA* gene) [15–17] where mutation in index case was known.

Results

Table 2 demonstrates the normal range of lysosomal enzymes expressed in various prenatal tissues where normal child was delivered and a follow-up study was carried out till 1 year of age confirming the normal status. Test results were compared using them as a reference range.

The preponderance of the diagnosis in the index case was observed for glycolipid or sphingolipid degradation (46 %) followed by glycosaminoglycans (GAGs) degradation (mucopolysaccharidoses 27.6 %), sulfatide degradation (13.5 %), glycogen degradation (Pompe disease 5.6 %), protein degradation (NCL-II 1.4 %), and defect in lysosomal trafficking proteins [mucolipidosis-II/III (ML-II/III) 5 %]. Of 140 prenatal cases studied, 60 (42.8 %) pregnancies were found to be affected with various LSDs with enzyme activity of 0–10 %. Thirteen (9.2 %) pregnancies have shown the carrier status with approximate intermediate enzyme activity expression of (~50 % of mean), while 67 (47.8 %) pregnancies have shown normal enzyme activity in Table 2.

From the affected fetuses, 5 (3.5 %) were affected with Niemann–Pick disease-A/B (NPD-A/B), 6 (4.3 %) with GM1 gangliosidosis, 3 (2.1 %) with Tay-Sachs disease, 2 (1.4 %) with Sandhoff disease, 1 (0.7 %) with Gaucher disease, 11 (7.8 %) with Hurler disease (MPS-I), 3 (2.1 %) with Hunter disease (MPS-II), 1 (0.7 %) with Sanfilippo type-A (MPS-IIIA), 2 (1.4 %) with Sanfilippo type-B (MPS-IIIB), 4 (2.8 %) with Morquio-A (MPS-IVA), 2 (1.4 %) with Maroteaux–Lamy syndrome (MPS-VI), 2 (1.4 %) with metachromatic leucodystrophy (MLD), 7 (5.0 %) with Krabbe disease, 5 (3.5 %) with Pompe

Table 1 Activity of lysosomal enzymes (nmol/h/mg protein) in various tissues (CV, CCV, CAF) during PD

Type of disorders	Enzyme estimated	Diagnosis of fetus									Total
		Affected fetus ($n = 60$)			Carrier fetus ($n = 13$)			Normal fetus ($n = 67$)			($n = 140$)
		CV	CCV	CAF	CV	CCV	CAF	CV	CCV	CAF	
Defects in degradation of glycol or sphingolipids											
Niemann–Pick disease A/B (NPD-A/B)	Sphingomyelinase	0.9	3.1 0.6 3.4	4.6	–	–	6.86	$n = 1$	$n = 2$	$n = 4$	$n = 13$
GM1 gangliosidosis	β-Galactosidase	6.4	2.7 23.8	22.3 17.2 8.2	–	–	–	$n = 1$[a]	$n = 1$[a]	$n = 7$	$n = 14$
GM2 gangliosidosis (Tay-Sachs disease)	Hex-A/hexosaminidase-T	21.7[b]/5,479.3	4.3/7,440.0	23.4[b]/4,454.5	–	–	247.2[b]/4,850.0 187.4/5,002.0	$n = 5$[a] $n = 1$	$n = 5$[a] $n = 2$[b]	$n = 5$ $n = 1$[b]	$n = 19$
GM2 gangliosidosis (Sandhoff disease)	Hex-A/hexosaminidase-T	43.5/408.2	–	32.1/62.5	66.66/895.7[a] 255.4/5,344.1	58.3/783.1[a] 54.54/708.0	189.7/2,114.2	–	–	$n = 4$	$n = 10$
Gaucher disease	β-Glucosidase	19.7[a]	13.0[a]	–	128.3	–	–	$n = 2$[a] $n = 1$	$n = 2$[a] $n = 1$	$n = 3$	$n = 9$
Defects in degradation of mucopolysaccaridesl											
Hurler disease (MPS-I)	α-Iduronidase	3.5 0.0[a]	0.0[a]	6.4 10.4 0.0 0.0 3.3 13.8 7.9 11.6 19.3	–	–	–	$n = 2$	–	$n = 5$	$n = 18$
Hunter disease (MPS-II)	α-Iduronidase-sulfatase	–	5.7	8.3 8.4	–	–	–	–	–	$n = 1$	$n = 4$
Sanfilippo-A (MPS-IIIA)	Heparan sulphamidase	0.9[a]	2.19[a]	–	–	–	–	–	–	–	$n = 1$
Sanfilippo-B (MPS-IIIB)	*N*-Ac-α-glucosaminidase	4.22	–	9.7	–	–	–	–	–	$n = 2$	$n = 4$
Morquio A (MPS-IVA)	β-Galactose-6-sulphate sulphatase	7.2[a] 6.5[a]	7.4[a] 6.7[a]	6.7 7.8	–	–	9.8	–	–	–	$n = 5$
Maroteaux–Lamy syndrome (MPS-VI)	Arylsulfatase-B	–	–	0.57 0.66	–	–	–	$n = 2$[a]	$n = 2$[a]	$n = 1$	$n = 5$
Sly syndrome (MPS-VII)	β-Glucuronidase	–	–	–	–	–	–	–	$n = 1$	$n = 1$	$n = 2$

Table 1 continued

Type of disorders	Enzyme estimated	Diagnosis of fetus									Total
		Affected fetus ($n = 60$)			Carrier fetus ($n = 13$)			Normal fetus ($n = 67$)			($n = 140$)
		CV	CCV	CAF	CV	CCV	CAF	CV	CCV	CAF	
Defects in degradation of sulfatides											
Metachromatic leucodystrophy (MLD)	Arylsulfatase-A	0.13[a]	0.4[a]	0.9	–	–	1.9	$n = 1$ $n = 1$[a]	$n = 1$[a]	$n = 4$	$n = 9$
Krabbe disease	β-Galactocerebrosidase	3.4	1.69 6.0 5.65 6.72	2.5 2.1	–	–	–	–	–	$n = 3$	$n = 10$
Defects in degradation of glycogen											
Pompe disease	α-1,4-Glucosidase	6.5 4.0[a]	6.3 3.4[a] 7.2	4.0	–	–	19.8	–	–	$n = 2$	$n = 8$
Defects in degradation of glycoproteins											
Batten disease (NCL-II)	Tripeptidyl peptidase-1	–	–	10.2 3.3	–	–	–	–	–	–	$n = 2$
Defects in lysosomal of trafficking proteins											
Mucolipidosis-II/III (ML-II/III)	β-Glucuronidase Hexosaminidase T β-Galactosidase	–	–	21.19 26.4 11.9 17.2 759.2 444.2 641.2 553.0 65.7 37.9 37.5 22.5	–	–	45.0 37.7 – – 1,908.3 1,943.0 – – 71.0 203.2 – –	–	–	$n = 1$	$n = 7$

[a] Enzyme study was carried out in both tissues—CV and CCV in the same patient

[b] Molecular study was carried out in prenatal sample

Table 2 Normal values of lysosomal enzymes in various biological tissues

Deficient enzymes (units)	Normal ranges		
	Uncultured CV (CV)[a]	Cultured CV (CCV)[a]	Cultured amniotic fluid cells (CAF)[a]
Sphingomyelinase (nmol/h/mg protein)	7.95–13.61 (8)	13.3–18.8 (4)	17.0–69.4 (8)
Hexosaminidase-A (nmol/h/mg protein)	298–929.0 (10)	271–1,621 (8)	271–2,213.2 (9)
Hexosaminidase-T (nmol/h/mg protein)	1,000–1,728 (13)	4,507–15,473.2 (15)	4,487.0–16,286.2 (25)
β-Galactosidase (nmol/h/mg protein)	131.28–501.0 (6)	150–644 (25)	296–1,195 (30)
β-Glucosidase (nmol/h/mg protein)	90–198 (9)	115–409 (18)	118–584.8 (30)
α-Iduronidase (nmol/h/mg protein)	12.0–32.5 (11)	19.27–93.4 (10)	85.5–156.3 (14)
α-Iduronidase-sulfatase (nmol/4 h/mg protein)	28.1–48.0 (3.0)	218.7–372.0 (4)	63.1–261.1 (17)
Heparan sulphamidase (nmol/17 h/mg protein)	2.5–8.0 (3)	10.2–25.3 (3)	ND
N-Ac-α-glucosaminidase (nmol/h/mg protein)	ND	ND	21–73.3 (6)
β-Galactose-6-sulphate sulphatase (nmol/17 h/mg protein)	19.4–22.5 (3)	20.8–38.9 (3)	16.8–25.1 (5)
Arylsulfatase-B (nmol/h/mg protein)	1.3–3.8 (3)	2.6–4.75 (4)	3.5–8.1 (6)
β-Glucuronidase (nmol/h/mg protein)	91.04–135.13 (8)	26.5–149.6 (11)	50.07–197.1 (13)
Arylsulfatase-A (nmol/h/mg protein)	1.08–6.0 (4)	3.5–8.0 (5)	3.0–6.0 (9)
β-Galactocerebrosidase (nmol/17 h/mg protein)	20.8–56.8 (4)	21.9–99.5 (4)	20.1–74.93 (10)
α-1,4-Glucosidase (nmol/h/mg protein)	92.2–116.9 (3)	30.0–90.8 (8)	28.6–75.0 (10)
Tripeptidyl peptidase-1 (nmol/h/mg protein)	ND	59.1–78.8 (3)	27.3–306 (10)

Number of cases investigated under each category is shown in parenthesis

[a] Number of cases studied from CCV includes CV cells while those with CAF have not been studied from CV or CCV

disease, 2 (1.4 %) with Batten disease (NCL-II) and 4 (2.8 %) with ML-II/III. Intermediate enzyme activity (~50 % of mean) was detected in 13 (9.2 %) pregnancies that includes 4 (2.8 %) fetuses with Sandhoff disease, 2 (1.4 %) with Tay-Sachs disease, 2 (1.4 %) with ML-II/III, each 1 (0.7 % for each) with Gaucher, MLD, NPD-A/B and Pompe disease. Except one case of MPS-IVA which was found to be a carrier (~30 % enzyme activity) during PD and was found to be affected postnatally.

Molecular analysis was carried out in seven pregnancies followed by enzyme study with known mutation in the index case. This was carried out for Tay-Sachs disease in six pregnancies, of which two were affected. Homozygous mutation for 4 bp insertion at c.1277_1278insTATC (p.Y427IfsX5) and c.320G>A (p.E114K) one each was detected in enzymatically confirmed affected fetus same as seen in the index case. Enzymatically proven carrier subject ($n = 1$) showed only one copy of mutation c.964G>A (p.D322N). In remaining three case subjects, absence of mutation [c.964G>A (p.D322N), c.964G>A (p.D322N)/c.1277_1278insTATC (p.Y427IfsX5) and c.1454G>A (p.W485X)] in *HEXA* gene further supports the enzymatic results (Fig. 1). Mutation analysis of Gaucher disease carried out in a case during PD showed carrier status for c.1448T>C (p.L444P or p.L483P) mutation in *GBA* gene same as carrier (Fig. 2).

Discussion

The present study demonstrates the utility of fetal tissue in the PD of various lysosomal disorders. Various combinations such as CV, CCV and CAF were used in the study. Total 140 cases were investigated and 60 (42.9 %) were found to be affected, 13 (9.2 %) were carrier and 67 (47.8 %) were normal. In 19 cases, CCV were processed in addition to CV and comparable results were observed for affected, carrier and normal in all the case subjects except one. This further confirms the previous report about fetal tissue that can be utilized for the diagnosis of various LSDs using lysosomal enzyme during PD. [1, 2, 18].

None of the cases has shown any difference in enzyme activity in CV or CCV cells in any of the 19 subjects which further suggests that direct CV cells can be used reliably for the rapid PD of LSDs. However low activity of α-iduronidase has been reported in uncultured CV cells as compared to CCV cells [19] and doubts have been expressed for the reliability of using only uncultured CV cells for Hurler disease. Although Young [20] has observed CV cells to be highly reliable in 24 pregnancies affected by Hurler disease, caution must be taken in cases where high residual activity is detected in CV. This needs further confirmation either by CCV cells or CAF cells as detected in one of our case. Initially CV and CCV both were processed for MPS-IVA

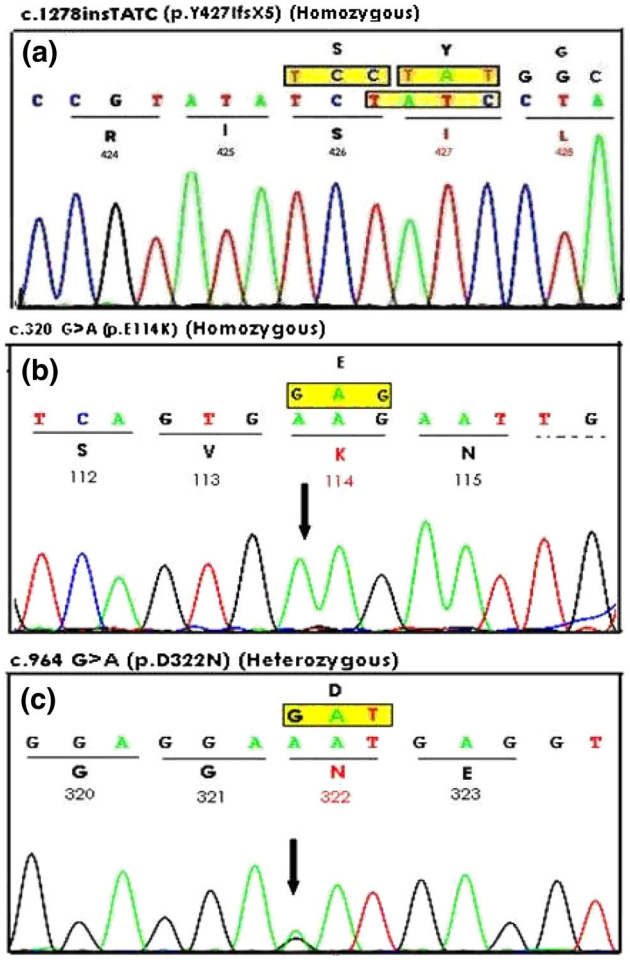

Fig. 1 a–c Chromatogram of *HEXA* gene mutations. **a** c.1277_1278insTATC (p.Y47IfsX5, homozygous), **b** c.320G>A (p.E114K, homozygous), and **c** c.964G>A (p.D322N, heterozygous)

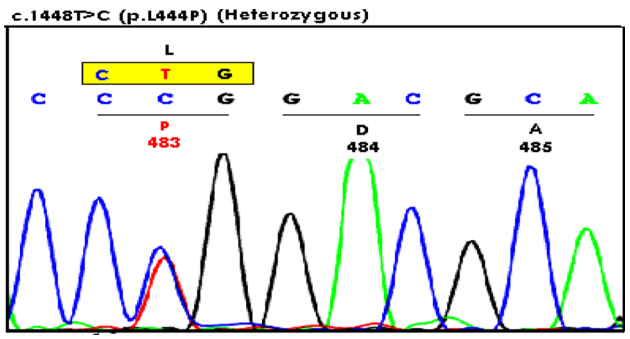

Fig. 2 Chromatogram of c.1448 (p.L444P) mutation in *GBA* gene (heterozygous)

where 30 % enzyme activity was detected and was considered as carrier but on follow-up after delivery, it was found to be affected. This indicates that in MPS cases, where residual activity of enzyme in CV cells is high, AF study by GAG excretion pattern in combination with CAF cells could be the preferred choice as suggested by Lake et al. [1]. They could make PD based on AF–GAG analysis followed by enzyme study from CAF cells. Moreover, Zhang et al. [21] also could make the prenatal confirmative diagnosis of MPS-III (Sanfilippo A/B) based on AF–GAG study.

Intermediate activity of hexosaminidase-A (Hex-A) with normal total-hexosaminidase (Hex-T) activity was detected in two pregnancies suggesting carrier status for Tay-Sachs disease (GM2 gangliosidosis). Due to overlapping activity of Hex-A in B1 variant of GM2 gangliosidosis, the authors used synthetic substrate (4-methylumbelliferyl β-*N*-acetyl glucosamine-6-sulphate) instead of heat inactivation, where Hex-A remains low in B1 variant [1]. This could perhaps minimize the isoenzyme interference in GM2 gangliosidosis which is commonly observed in these cases and those with MLD and Maroteaux–Lamy syndrome (MPS-VI) [22].

Multiple enzyme deficiency is the gold standard for the diagnosis of ML-II/III. AF supernatant can be used as an initial screening test to measure enzyme activity which gets elevated in an affected pregnancy with ML-II/III disease [23]. None of the cases was studied for direct enzyme study from AF. In the present study, out of seven cases suspected with ML-II/III, four were confirmed to have low activity of multiple enzymes in CAF especially β-glucuronidase, β-Hex-T and β-galactosidase while in remaining three cases two were shown intermediate enzymes activities and one was normal which was confirmed postnatally. However Besley et al., carried out AF-supernatant study from a fetus affected with I-cell diseases (ML-II) for arylsulfatase-A, β-Hex-T and β-glucuronidase, but in another pregnancy from a different family the AF enzyme activities were not raised, though the activities in CAF cells were much less consistent with that of an affected fetus and conclude that AF supernatant after 14.5 weeks gestation would show abnormally high enzyme activities in an affected I-cell pregnancy. Though, normal AF supernatant results should always be confirmed by studying CAF in pregnancies suspected for ML-II/III [23].

In the present study molecular analysis was carried out in seven cases which include six Tay-Sachs and one Gaucher disease with known mutation and all were in concordance with the enzymes study. It can be affirmed that enzymes study from prenatal fetal tissue is as specific and sensitive as molecular study. Nonetheless mutation detection in the index case is the prerequisite for this.

Although, enzyme based PD for some of the lysosomal disorders often pose a problem of pseudo-deficiencies of few of the lysosomal enzymes [24]. In such cases, mutation analysis as well as enzyme activity is necessary in the parents and the index case before offering PD. The failure to study the presence of a pseudo-deficiency allele in a

family could lead to an incorrect PD, which is a major limitation of the study. Another confirmative approach is to use biochemical analysis and morphology of CV tissue, which has been successfully used at Great Ormond Street Hospital for Children in approximately 1,000 pregnancies at risk for LSDs [25].

Selection of the biological material to be used for PD is always a dilemma for clinician due to fetal safety and variable enzymatic activity in different tissues. In most of the studies done so far for PD of LSDs, enzyme activities were measured either from CV, CCV cells, or CAF cells. CV is the most preferred one owing to benefit of performing direct biochemical analysis with rapid results as compared to cultured amniotic cells that can take days to weeks to provide enough material for biochemical testing. Nevertheless, experience also suggests that the use of CV (uncultured and/or cultured) provides an early result with a safety to the mother's decision while deciding for the termination, in case of an affected pregnancy. The CAF cells can be offered where probability of obtaining material in early pregnancy is less and in cases where CV cells fail to grow in vitro or enzyme activities are poorly expressed where heterozygous and affected status of the fetus is not clear. However, AF is preferred over CV study in pregnancies requiring PD for various MPS disorders and I-cell disease as direct AF study for various enzymes and excretion pattern of chondroitin sulfate, heparan sulfate and dermatan sulfate can be performed as a prenatal screening test. Based on this, confirmative study can be carried out from the cultured cells by carrying out selective lysosomal enzyme investigation.

To conclude, PD of LSDs can be carried out by enzymes study from CV/CCV and CAF with an accuracy of molecular method. However, in cases of MPS and ML-II/III, AF is preferred over CV/CCV. In addition, special care is needed while interpreting enzyme results encompassing carrier status and need to be further evaluated by molecular study.

Acknowledgments Our sincere thanks to Dr. Mamta Muranjan, Dr. Aparna Kulkarni and Dr. Jigish Trivedi for references. This study was partly supported by ICMR [54/2/2005-BMS] and FRIGE.

Conflict of interest None.

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Molecular Genetics and Metabolism Reports 1 (2014) 425–430

Contents lists available at ScienceDirect

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Expanding the spectrum of *HEXA* mutations in Indian patients with Tay–Sachs disease

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ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 11 September 2014
Accepted 11 September 2014
Available online 29 September 2014

Keywords:
Tay–Sachs disease
HEXA gene
β-Hexosaminidase-A
Lysosomal enzyme

ABSTRACT

Tay–Sachs disease is an autosomal recessive neurodegenerative disorder occurring due to impaired activity of β-hexosaminidase-A (EC 3.2.1.52), resulting from the mutation in *HEXA* gene. Very little is known about the molecular pathology of TSD in Indian children except for a few mutations identified by us. The present study is aimed to determine additional mutations leading to Tay–Sachs disease in nine patients confirmed by the deficiency of β-hexosaminidase-A (<2% of total hexosaminidase activity for infantile patients) in leucocytes. The enzyme activity was assessed by using substrates 4-methylumbelliferyl-N-acetyl-β-D-glucosamine and 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulfate for total-hexosaminidase and hexosaminidase-A respectively, and heat inactivation method for carrier detection. The exons and exon–intron boundaries of the *HEXA* gene were bi-directionally sequenced on an automated sequencer. 'In silico' analyses for novel mutations were carried out using SIFT, Polyphen2 and MutationT@ster software programs. The structural study was carried out by UCSF Chimera software using the crystallographic structure of β-hexosaminidase-A (PDB-ID: 2GJX) as the template. Our study identified four novel mutations in three cases. These include a compound heterozygous missense mutation c.524A>C (D175A) and c.805G>C (p.G269R) in one case; and one small 1 bp deletion c.426delT (p.F142LfsX57) and one splice site mutation c.459+4A>C in the other two cases respectively. None of these mutations were detected in 100 chromosomes from healthy individuals of the same ethnic group. Three previously reported missense mutations, (i) c.532C>T (p.R178C), (ii) c.964G>T (p.D322Y), and (iii) c.1385A>T (p.E462V); two nonsense mutations (i) c.709C>T (p.Q237X) and (ii) c.1528C>T (p.R510X), one 4 bp insertion c.1277_1278insTATC (p.Y427IfsX5) and one splice site mutation c.459+5G>A were also identified in six cases. We observe from this study that novel mutations are more frequently observed in Indian patients with Tay–Sachs disease with clustering of ~73% of disease causing mutations in exons 5 to 12. This database can be used for a carrier rate screening in the larger population of the country.

1. Introduction

Tay–Sachs disease (TSD) (MIM# 272800) is the second most common lipid storage disorder among the majority of lysosomal storage disorders (LSDs) studied in India [1]. It is an autosomal recessive neurodegenerative disorder caused due to β-hexosaminidase-A (Hex-A) deficiency owing to a mutation in the *HEXA* gene (MIM* 606869). The gene encodes α-subunit of β-hexosaminidase-A, a lysosomal enzyme composed of α- and β-polypeptides [2]. The resulting phenotype of TSD can be variable with an acute infantile form of early onset leading to rapid neuroregression and early death to a progressive later onset form compatible with a longer survival. Clinical features include the presence of neuroregression, generalized hypotonia, exaggerated startle response, and a cherry-red spot on the fundus. Affected patients have a deficient enzyme activity of β-hexosaminidase-A (Hex-A) in leukocytes/plasma or fibroblasts [3]. The human *HEXA* gene is located on chromosomes 15q23–q24 encompassing 14 exons. As per HGMD (human gene mutation database), nearly 134 mutations have been reported so far in the gene to cause TSD (http://www.hgmd.cf.ac.uk/).

Abbreviations: TSD, Tay–Sachs disease; SD, Sandhoff disease; Hex-A, β-Hexosaminidase A; LSDs, Lysosomal storage disorders; MUG, 4-Methylumbelliferyl-N-acetyl-β-D-glucosamine; MUGS, 4-Methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulfate.

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http://dx.doi.org/10.1016/j.ymgmr.2014.09.004

Majority of these mutations were private mutations present in a single or few families. Only few mutations have been commonly reported for TSD in particular ethnic communities or geographically isolated populations. In Ashkenazi Jews, one of the three common mutations i.e. c.1277_1278insTATC, c.1421+1G>C and c.805G>A (p.G269S) has been reported in 94–98% affected patients [4,5]. The c.1277_1278insTATC and the c.805G>A (p.G269S) mutations are also commonly found in non-Ashkenazi Jewish populations, along with the intron 9 splice site mutation (c.1073+1G>A), 7.6 kb French Canadian deletion [6], and c.571-1G>T in Japanese patients [2]. About 35% non-Jewish individuals also carry one of the two pseudo-deficiency alleles: c.739C>T (p.R247W) and c.745C>T (p.R249W), which are associated with no clinical phenotypes [7]. Although the mutation spectrum for Tay–Sachs disease in India has been partly addressed [1,8], our research continues to understand the molecular pathology of the disease. The present study was planned to identify additional novel and/or known mutations that will provide an insight into the molecular mechanism of the *HEXA* gene in Indian subjects.

2. Material and methods

2.1. Subject and sample collection

Nine families were included in the study. The Institutional ethics committee approved the study. For blood collection, clinical details were filled up in the case record forms after an informed written consent was obtained from the guardian of the study subjects. The clinical inclusion criteria were the presence of seizures, cherry red spots, neuroregression, exaggerated startle, and hypotonia followed by the confirmation of enzyme deficiency of Hex-A and mutation identification. Though neuroimaging criteria were not strictly followed up, it mainly included the presence of gray matter disease as shown by hypointensities in T2W images of the brain MRI.

2.2. Enzyme assay

Six milliliter peripheral blood was collected in an EDTA vacutainer from all the patients for leukocyte enzyme assay and DNA extraction. The enzyme activity was determined by the fluorimetric method using a specific synthetic substrate. The total-Hex activity was measured from the hydrolysis of the synthetic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine (MUG) that releases fluorescent 4-methylumbelliferone when acted upon by β-hexosaminidase. Hexosaminidase B (Hex-B) was determined as the activity left over after incubating the sample for 3 h at 50 °C. This procedure led to the loss of heat-labile Hex-A but not Hex-B or intermediate isoenzyme. Thus Hex-A activity was obtained after subtracting Hex-B activity from the total-Hex activity. Hex-A was also assayed with a more specific sulfated substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulfate (MUGS) [9]. Hex-A activity (obtained from MUGS lysis) was expressed as the percentage of total-Hex activity (obtained from MUG lysis).

2.3. Molecular analysis of HEXA gene by sequencing

Genomic DNA was extracted by the standard salting out method [10]. The exonic and intronic flanking sequences of the *HEXA* gene were PCR amplified in 14 fragments using the primer pairs as described earlier [11]. Amplification included 5-min denaturation at 94 °C followed by 30 cycles each consisting of 1 min denaturation at 94 °C for 45 s of annealing at temperature ranging from 60 to 65 °C, depending on the characteristic of each exon (melting temperature), and 45 sec extension at 72 °C. Final extension was carried out at 72 °C for 10 min. The negative control contained all the above ingredients except DNA. PCR products were electrophoresed on 2% agarose along with the appropriate negative controls and 100 base-pair DNA ladder. Products that passed this quality check were purified by treatment with Exo-SAP-IT™ (USB Corporation, OH, USA) or Column purification and then sequenced using BigDye Terminator v3.1. Capillary electrophoresis was performed on an automated sequencer ABI-3130 (Applied Biosystems, CA, USA) and nucleotide numbers are derived from *HEXA* cDNA sequences (RefSeq NM_000520.4). Putative mutations were confirmed in two separate PCR products from the patient's DNA. Heterozygosity for these mutations was confirmed in parents. The mutations identified were then looked up in public databases such as The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk), SNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html) and McGill University database (http://www.hexdb.mcgill.ca). Novel variants were further confirmed as pathogenic by studying 50 control individuals with normal leukocyte enzyme results.

2.4. In silico analysis

Prediction of functional effects of non-synonymous single nucleotide substitutions (nsSNPs) was done using software SIFT (Sorting Intolerant From Tolerant) (available at http://sift.jcvi.org/), Polyphen2 (Polymorphism Phenotyping v2) (available at http://genetics.bwh.harvard.edu/pph2/) and MutationT@ster (available at http://www.mutationtaster.org/) [12–14]. HumVar-trained prediction model of Polyphen2 was used to distinguish mutations with drastic effects from all the remaining human variations, including many mildly deleterious alleles. Evolutionary conservation of the amino acid residues of Hex A was analyzed using ClustalW program available online at (http://www.uniprot.org/help/sequence-alignments).

2.5. Structural studies

Structural study of two missense mutant alleles was carried out by UCSF Chimera software using the crystallographic structure of Hex-A (PDB ID: 2GJX) as the template. The Root Mean Square Deviation (RMSD) of the mutant structures with respect to the wild-type structure was calculated. RMSD values of more than 0.15 were considered as significant structural perturbations that could have functional implications for the protein [15].

3. Results

Consanguinity was present in 4/9 (44.4%) patients. The mean age at presentation was 12.3 months (±5.3). All the cases were classified as infantile as they had a history of neuroregression since infancy. All the cases were presented with seizures, neuroregression, cherry red spot on fundus, exaggerated startle, hypotonia, and brisk deep tendon reflexes. However, none of the cases had hepato-splenomegaly. The CT/MRI brain was available in 4/9 cases and showed characteristic findings of decrease in thalami and decreased attenuation of basal ganglia isodense with white matter, and few cases had dysmyelination.

We confirmed 7/9 cases with TSD [deficient activity of β-hexosaminidase-A (Hex-A) in leucocytes], with carrier detection (% Hex-A) in 2 parents where proband was not alive but was earlier diagnosed as a TSD by enzyme study (Table 1). Molecular analysis was carried out in all the 9 affected families with TSD followed by bi-directional sequencing for common mutation screening. Exon sequencing analysis revealed 11 mutations in 9 patients, 4 of which were novel including compound heterozygous missense mutations c.524A>C (p.D175A) and c.805G>C (p.G269R) in exons 5 and 7 respectively, one small 1 bp deletion c.426delT (p.F142LfsX57) in exon 4 and one patient was a compound heterozygote for novel splice site mutation c.459+4A>C and known missense mutation c.532C>T (p.R178C). In addition, previously known missense mutations c.964G>T (p.D322Y) in exon 9, two nonsense mutations c.709C>T (p.Q237X) and c.1528C>T (p.R510X) in exons 7 and 14 respectively and one insertion mutation c.1277_1278insTATC (p.Y427IfsX5) were detected. One patient was found to be heterozygous for c.1385A>T (p.E462V) with unknown

Table 1
Clinical, biochemical and molecular details of the Indian patients with Tay-Sachs disease.

Patient ID	Age at diagnosis	Consanguinity	Hex-A activity (MUGS) (nmol/h/mg) = (x)	Total Hex activity (MUG) = (y)	Hex A % = (x / y) × 100	MUG/MUGS ratio[b]	Genotypes	
							Mutations at DNA level	Mutations at protein level
1.	1.3 yr	Yes	9.3	2250.3	0.4	1.50	c.1277_1278insTATC/c.1277_1278insTATC	p.Y471fsX5/p.Y471fsX5
2.	1.5 months	No	8.9	2254.3	0.39	1.44	c.964G>T/c.964G>T	p.D322Y/p.D322Y
3.	11 months	Yes	10.8	1273.2	0.84	3.1	c.1528C>T/c.1528C>T	p.R510X/p.R510X
4.	1.1 yr	No	7.0	2602.7	0.27	1.0	c.524A>C/c.805G>C	p.D175A/p.G269R
5.	1.5 yr	No	0.00	1781.0	0.0	0.0	c.459+5G>A/c.459+5G>A	−/−
6.	1.5 yr	Yes	6.2	2090.0	0.29	1.07	c.426delT/c.426delT	p.F142Lfsx57/p.F142Lfsx57
7.	1 yr	No	7.9	2185.7	0.36	1.33	c.1385A>T/?	p.E462V/?
8.[a]	1 yr	Yes	0.9	761	0.11	0.41	−/−	[−/−]
			Mother HLC − 53.8%				Mother − [c.532C>T/normal]	Mother − [p.R178C/normal]
			Father HLC − 58.8%				Father − [c.459+4A>C/normal]	Father − [−/−]
9.[a]	1.3 yr	No	1.5	1526.3	0.098	0.36	−/−	[−/−]
			Mother HLC − 50.5%				Mother − [c.709>T/normal]	Mother − [p.Q237X/normal]
			Father HLC − 48.8%				Father − [c.709C>T/normal]	Father − [p.Q237X/normal]

Normal total-hexosaminidase values using MUG substrate in our controls − 723 to 2700 nmol/h/mg protein, normal hexosaminidase A levels − 62 to 77%; and normal MUGS activity 80 to 410 nmol/h/mg.
[a] DNA of index case is not available, HLC−heat labile activity in carrier parents.
[b] The MUG/MUGS ratio for Hex A is 3.7:1 [19].

second allele and another patient with splice site homozygous mutation c.459+5G>A in intron 4 as shown in Table 1 and Fig. 1. The novel mutations were not found in 100 control individuals from the same ethnic background.

The SIFT index, Polyphen2 scores and MutationT@ster scores for the non-synonymous single nucleotide substitutions are presented in Table 2. The predictions of the SIFT, Polyphen2 and MutationT@ster algorithms were in concordance with the observed pathogenicity in the case of mutations p.D175A, p.G269R, p.D322Y and p.E462V as described in Table 2. The RMSD (Root Mean Square Deviation) values for the modeled mutants were significant for the pathogenicity of all missense mutations (Table 2).

4. Discussion

The clinical features of infantile Tay–Sachs disease seen in our set of patients were consistent with the defined phenotype, except for the presence of prominent Mongolian spots on the back even beyond infancy in two patients. However, Mongolian spots are also reported in association with many of the patients with other LSDs [1,16] and in our case it could be an accidental finding. The enzyme activity was in accordance with the previous observations of infantile TSD cases [17,18]. However, MUG substrate is cleaved by all three isozymes and therefore is used to measure total-Hex activity. MUGS substrate is cleaved primarily by the α-subunit containing isozymes (Hex-A and S). However, the β active site can slowly hydrolyze this substrate with MUG/MUGS ratios of ~300:1 for Hex-B and 3.7:1 for Hex-A [19]. Thus each of the %-residual Hex-A values reported in Table 1 is multiplied by 3.7 and is varying from 0% to 3.1%, which is in accordance with the previously reported value.

Among 11 mutations demonstrated here, six have been previously reported in different populations that include missense mutations p.E462V and p.D322Y from the Indian population [1,8], p.R178C from Czechoslovakia [20], c.1277_1278insTATC from Indian, Ashkenazi Jews and non-Jewish populations [8,20], nonsense mutation p.R510X from Saudi Arabia [21] and Iranian population [22], and splice site mutation c.459+5G>A from Argentinean [23] and Spanish populations [24]. The nonsense mutation p.Q237X was previously reported as benign in accordance with the NCBI SNP database. However, a subunit lacking its last 293 C-terminal residues is highly likely to make the protein non-functional. In the present study this mutation was observed in both parents with the history of an affected child with TSD. Though, mutation study in the affected child could not be carried out due to sudden death and unavailability of a sample. The National Center for Biotechnology Information's (NCBI) single nucleotide polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs150675340) reveals that the minor allele count and pathogenic potential of this polymorphism (rs150675340) are not available. Generally, nonsense and frame-shift mutations result in the reduction of mRNA in the *HEXA* gene. Diminished amounts of mRNA have been reported in several mutations in the *HEXA* gene [25]. The presence of one mutant and one normal copy of this allele in both the parents suggests that the child would have had the mutation in the homozygous state, thus being responsible for causing of the disease.

In the present study, novel mutations p.D175A and p.G269R were found as compound heterozygous in infantile TSD. The missense mutation p.D175A disrupts the β-sheet due to an amino acid change from a positively charged aspartic acid to a hydrophobic alanine residue at codon 175 of exon 5. At the same time, p.G269R mutation creates over-packing and backbone disruption due to the replacement of a small hydrophilic group (glycine) with a positively charged residue (Alanine) at codon 269 of exon-7 [Fig. 2(a)–(b)]. Therefore, disruption of the backbone from αGly269Arg may result in the movement of αHis262, with the likelihood of disrupting the coordination of residues in the active site. The RMSD of the modeled mutant is also significantly

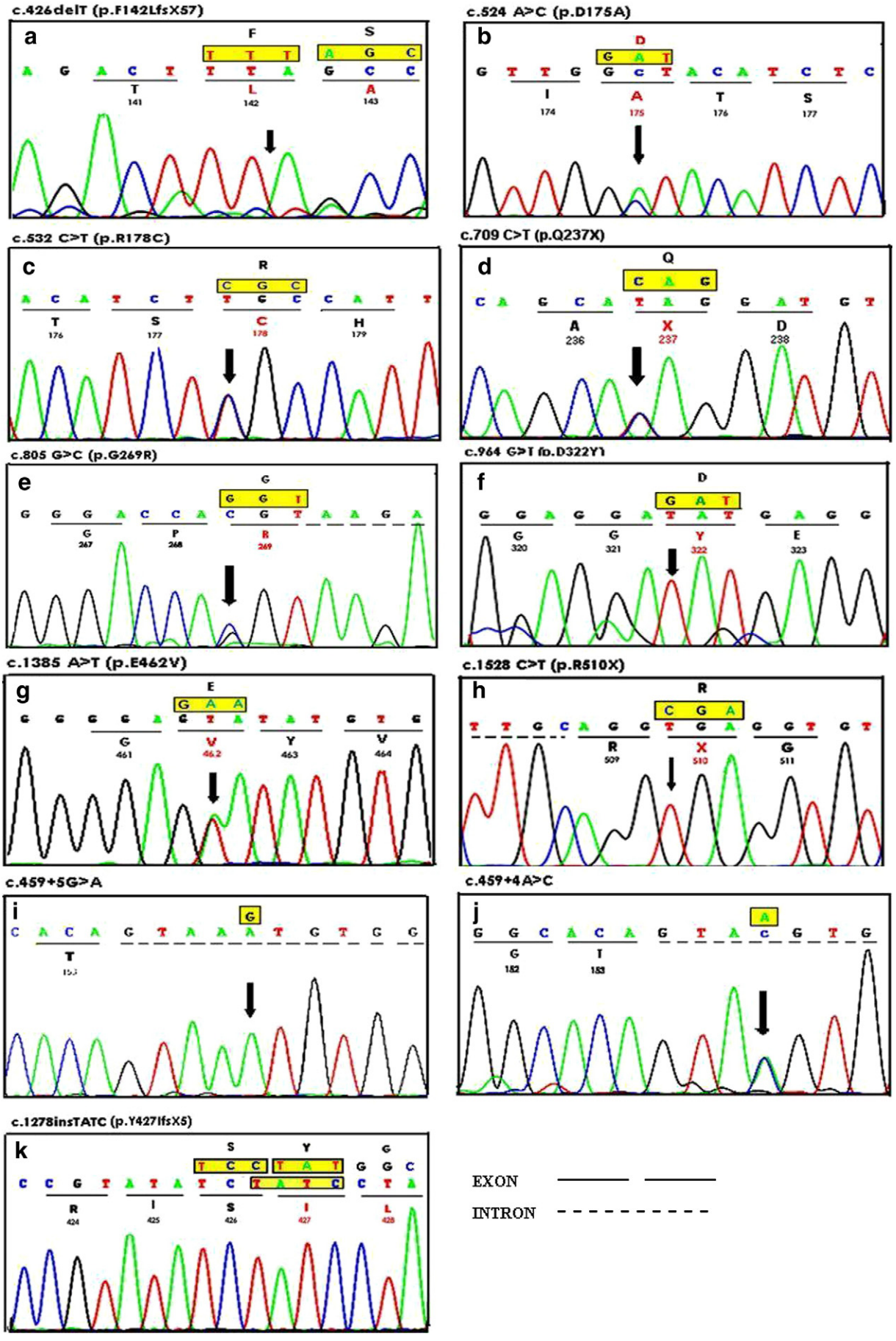
c.426delT (p.F142LfsX57)
a
c.524 A>C (p.D175A)
b
c.532 C>T (p.R178C)
c
c.709 C>T (p.Q237X)
d
c.805 G>C (p.G269R)
e
c.964 G>T (p.D322Y)
f
c.1385 A>T (p.E462V)
g
c.1528 C>T (p.R510X)
h
c.459+5G>A
i
c.459+4A>C
j
c.1278insTATC (p.Y427IfsX5)
k
EXON
INTRON

Table 2
Details of *HEXA* missense mutations detected in infantile TSD patients and in silico analysis.

Location	Codon number	Codon change	Amino acid change	MutationT@ster score	SIFT score	Polyphen2 score (sensitivity, specificity)	RMSD between native and mutant structures	Amino acid change
Exon 5	175	GAT–GCT	Asp175Ala	3.44 (DC)	0.00 (IT)	1.00 (0, 1) (PD)	0.268, (PD)	Acidic to nonpolar
Exon 7	269	GGT–GCT	Gly269Arg	3.41 (DC)	0.00 (IT)	1.00 (0,1)(PD)	0.225, (PD)	Hydrophilic to charged
Exon 8	322	GAT–TAT	Asp322Tyr	4.36 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.178, (PD)	Non- cyclic to cyclic
Exon 12	462	GAA–GTA	Glu462Val	3.3 (DC)	0.00 (IT)	1.00 (0,1) (PD)	0.182, (PD)	Acidic to nonpolar

DC = disease causing, P = polymorphism, IT = intolerant, PD = probably damaging.
The MutationT@ster score is taken from an amino acid substitution matrix (Grantham Matrix) which takes into account the physico-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid which scores may range from 0.0 to 6.0.
The SIFT score is the normalized the probability that the amino acid change is tolerated and ranges from 0 to 1. The amino acid substitution is predicted damaging if the score id ≤ 0.05, and tolerated if the score is >0.05.
The Polyphen2 score is the naïve Bayes posterior to probability that this mutation is damaging and thus ranges from 0 to 1.

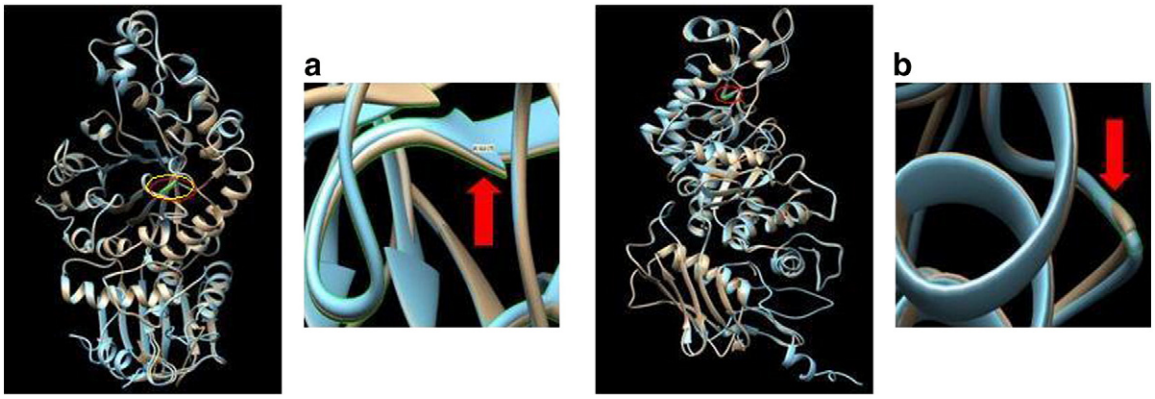

Fig. 2. Superimposed native structures (brown) and mutant structure (blue) produced using USCF Chimera software: (a) mutation p.D175A disrupts the β-sheet and (b) p.G269R creates overpacking and backbone disruption.

higher as compared to the wild-type protein (Table 2). Additionally, p.G269 is also the site of the mutation in late onset Tay–Sachs (LOTS) (p.G269S) with high frequency in the Ashkenazi Jews and results in an unstable alpha subunit precursor. This fails to associate with the beta subunit, suggesting that the mutation reported here, found in a patient with the infantile acute disease, has a more deleterious impact on the enzyme or its expression [15]. Protein sequence alignment demonstrated that p.D175A and p.G269R are highly conserved residues among different species. All these novel mutations were found to be pathogenic by SIFT, Polyphen2 and MutationT@aster softwares.

Another novel mutation (1 bp deletion c.426delT) was observed in exon 4 in an infantile TSD patient that changes the reading frame at codon 142 and introduces a premature stop at codon 199 (p.F142LfsX57), leading to a truncated protein or a nonsense-mediated mRNA decay (NMD). This mutation was also evaluated for pathogenicity using MutationT@ster and was found to be disease causing. Nonetheless, in a non-Jewish French child, a 2 bp deletion in the same codon (codon-142) is reported by Akli et al. [26].

In one case, the novel intronic mutation c.459+4A>C was found with previously reported mutation p.R178C in a compound heterozygous state. This mutation is in close proximity to the reported splice mutation c.459+5G>A in Argentinean and Spanish populations [23,24]. It was evaluated for pathogenicity using MutationT@ster and was found to be disease causing; and was predicted to lead to a loss of aberrant donor splice site at this position (score 0.87). The patient was not available for further functional study of the mutation. As per our knowledge, these novel mutations have never been identified in TSD patients from other ethnic groups.

A large number of gene mutations causing TSD have been previously reported in the *HEXA* mutation database (http://www.hexdb.mcgill.ca/). Among them, mutations resulting in gross alterations in the Hex α-subunit sequence are generally found in the severe infantile form. However, missense mutations causing amino acid substitutions have been found in both the infantile and late onset phenotypes [27]. The detrimental effect of most mutations is on the overall folding and/or transport of the protein rather than on the functional sites, as reported by Mahuran [28]. Some of the mutations identified in our series affect crucial active residues or cause significant structural aberrations leading to the infantile form of the disease.

5. Conclusion

Identification of four novel mutations provides additional insight into the molecular pathology of TSD in Indian subjects. Combining this with our previous study of 28 cases, mutations responsible for TSD in Indian patients are mostly novel with nearly 73% mutations clustering in between exons 5 to 12. This database can be used as a tool for the carrier screening of a larger Indian population.

Conflict of interest

The authors have declared that no competing interests exist.

Author contributions

JS was involved in the designing of the study, standardization of technical procedure, and preparation of manuscript and will also act

Fig. 1. Sequence chromatogram of *HEXA* gene mutations. (a): c.426delT (p.F142LFsX57) (homozygous); (b): c.524A>C (p.D175A) (heterozygous); (c): c.532C>T (p.R178C) (heterozygous); (d): c.709C>T (p.Q237X) (heterozygous); (e): c.805G>C (p.G269R) (heterozygous); (f): c.964G>T (p.D322Y) (homozygous); (g): c.1385A>T (p.E462V) (heterozygous); (h): c.1528C>T (p.R510X); (i): c.459+5G>A (homozygous); (j): c.459+4A>C (heterozygous); (k): c.1277_1278insTATC (p.Y47IfsX5) (homozygous).

as a guarantor. MM was involved in processing the sample, standardization of molecular techniques and preparation of the manuscript. FS have critically evaluated the manuscript. CD, MK, SP, HS, and UK were involved in clinical data collection. SG helped in technical support. All the authors have read and approved the manuscript.

Acknowledgments

We are grateful to all the referring clinicians and patients for their support; without their consent this study would not have been possible. We acknowledge the Indian Council of Medical Research (ICMR) for their financial support to conduct this study.

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